Title: Immune-modulation of two BATF3 paralogues in rainbow trout Oncorhynchus mykiss

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Abstract: Basic leucine zipper transcription factor ATF-like (BATF) -3 is a member of the activator protein 1 (AP-1) family of transcription factors and is known to play a vital role in regulating differentiation of antigen-presenting cells in mammals. In this study, two BATF3 homologues (termed BATF3a and BATF3b) have been identified in rainbow trout (Oncorhynchus mykiss). Both genes were constitutively expressed in tissues, with particularly high levels of BATF3a in spleen, liver, pyloric caeca and head kidney. BATF3a was also more highly induced by PAMPs and cytokines in cultured cells, with type II IFN a particularly potent inducer. In rIL-4/13 pre-stimulated cells, the viral PAMPS polyI:C and R848 had the most pronounced effect on BATF3 expression. BATF3 expression could also be modulated in vivo, following infection with Yersinia ruckeri, a bacterial pathogen causing redmouth disease in salmonids, or with the rhabdovirus IHNV. The results suggest that BATF3 may be functionally conserved in regulating the differentiation and activation of immune cells in lower vertebrates and could be explored as a potential marker for comparative investigation of leucocyte lineage commitment across the vertebrate phyla.
Dear Editor,

Please find out the revised version of the manuscript. We made substantial amendment according to reviewers' comments. These include additional data on the expression of BATF3a and BATF3b in RTS-11 cells treated with stimulants for 6 h and Western blotting showing the cross-activity of human anti-BATF3 polyclonal antibody with trout BATF3a and BATF3b expressed in bacteria. I hope you will find the revised manuscript suitable for publication in Molecular Immunology. Thank you for your consideration. I look forward to your decision.

Yours sincerely,

Jun Zou
Ref.: Ms. No. MIMM-D-17-00506

Immune-modulation of two BATF3 paralogues in rainbow trout Oncorhynchus mykiss Molecular Immunology

Dear Dr. Zou,

Thank you for submitting your manuscript to Molecular Immunology. Reviewers have now commented on your paper. Based on these comments and their own assessment, the editors consider your work appropriate for publication in Molecular Immunology, but there are a number of significant concerns that preclude acceptance of the manuscript in its present form. If you are prepared to undertake the work required to address these issues I would be pleased to consider a revised version for evaluation and possible publication.

For your guidance, reviewers' comments are appended below.

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Yours sincerely,

Victor Mulero, PhD
Associate editor
Molecular Immunology

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Reviewer #1: The manuscript describes the first characterization of BATF3 in fish, including evidences of its expression mainly in spleen of trout. The authors provide evidence on up-regulation of BATF3 in response to PAMPs, IFNg and, viral and bacterial challenge. The experimental results try to demonstrate the functional role of BETF3 in the immune response of trout. However, the conclusions require further experimental analyzes, or the authors could focus on a particular scope of the different aspects exposed.

To affirm that BATF3 is an immunomodulator the authors must provide experimental evidence that includes inhibition of BATF3 expression, such as silencing of the gene by siRNA or other appropriate technique. Without this type of analysis the manuscript only gives evidence of coincident gene expression, but not of causality.

Response: We agree with the reviewer that functional characterisation is needed to fully ascertain the roles of BATF3. This would be the focus of our future work in this area, especially as siRNA knock-down is not yet an established method in fish cells. In line with the reviewer’s comments we have revised the text so as not to exaggerate the findings, including deletion of the speculation on the functional roles of BATF3 in trout.
The experimental results of Figures 7 and 8 require a transcriptional analysis at different times, which occurs before 24 hours?

Response: We have now analysed the effects of TLR ligands in RTS-11 after 6 h stimulation. The results indeed show distinct induction patterns of BATF3a and BATF3b expression. These data are presented in Fig. 7E.

The response to IFNgamma could be evaluated with better evidence, such as expression of IFNγR1 / R2 receptors, or STAT 1, or some gene with GAS sequence (regulated by IFNgamma).

Response: The activities of trout recombinant IFN-γ have been previously evaluated by our group and others (Zou et al., 2005; Gao et al., 2009; Skjesol et al., 2010). The work published by Gao et al. (2009) and Skjesol et al (2010) has shown that rIFNγ induces expression of IFN-γR2 in RTG-2 cells and STAT1 phosphorylation in salmon head kidney cells and TO cells. We have added comments in the Materials and Methods and included additional references.

Minor

1. Authors must provide evidence of the purity of the all recombinant proteins used

Response: We have added a comment on the purity of recombinant proteins in the text and provided the relevant information.

2. In the introduction it is required to better description of BATF3 biochemistry, what is its molecular weight?

Response: The following information has been added in the Introduction: “...The genes encode a protein of 127 aa and 118 aa respectively, that share high homology (80% similarity), lack a signal peptide and bind to short nucleotide motifs in the promoter region of target genes.”

3. Introduction, what ligands activate the expression of BATF3 in higher vertebrates? exist evidences at the protein level?

Response: To answer the question about what ligands activate the expression of BATF3 in higher vertebrates, we performed an extensive literature search and could not find any published work.

4. Mat and met, 2.6 the protocol must be better explained, in the legend of figure 8 is better explained, IL4 / IL13A is a recombinant? (line 234, is rIL4 / IL13A)

Response: Materials and Methods 2.6 has been revised. IL-4/13A is now changed to rIL-4/13A in the manuscript.
5. the immunohistochemistry protocol must be improved ... blocking conditions? details of inhibition of endogenous peroxidase?

Response: In the IHC experiment, blocking was performed according to the protocol using the hydrogen peroxidase blocking solution supplied by Dako to inhibit the antibody cross reactivity with endogenous peroxidase activity. Prior to incubation with HRP-conjugated secondary antibody, tissues were incubated with peroxidase blocking solution for 7 minutes at room temperature. This ensures minimum staining in the negative control. Additional details are now added in the Materials and Methods.

6. Why do the authors use a spleen cell line and a primary head kidney (HKL) culture? (and not a primary spleen culture too?)

Response: The rainbow trout spleen cell line RTS-11 is a well characterised monocyte/macrophage like cell line. We felt it would be interesting to examine the expression of BATF3 in these cells. We did not use enriched primary spleen monocytes/macrophages simply due to the practical reason that very few attached monocyte/macrophage cells could be obtained.

7. Check in the text and figures that all recombinant molecules are indicated with "r".

Response: Thanks for the comment. We have revised the text accordingly.

8. It is necessary to indicate the exact homology percentage of the region which is recognizes the antibody against human BATF3 with the trout sequence.

Response: The peptide sequence of human BATF3 is not released by Merck, so it is not possible to provide the homology scores. However, the whole bZIP region of trout BATF3a and BATF3b share 78.5% and 83.1% similarity with the human counterpart. This information is in Fig. 3C.

9. The immunohistochemical picture is not clear, it also requires to include controls of the technique. It could also include pictures of immunocytochemistry of BATF3 in HKL or RTS11.

Response: Further information has been included to clarify the controls used. We feel that the immunocytochemical analysis of BATF3 in HKL or RTS-11 would not add any further useful information additional to the transcript analysis of gene expression.

Reviewer #2: In the current study, the authors describe the identification of two BATF3 homologues in rainbow trout. Additionally, they have performed a series of transcriptional analysis to establish how different stimuli affected the transcription of these factors both in vitro and in vivo. However,
the main problem is that the authors claim that this is the first identification of BATF3 genes in fish and this is not true.

Granja et al. described in 2015 the identification of CD8+ DC subset in rainbow trout skin that constitutively expressed BATF3. This is an important fact related to the work present here that is not mentioned at all throughout the paper despite it constituting a previous description of BATF3 genes in fish and the confirmation that BATF3 is used by a subset of fish DCs. The authors should include a reference to this paper in both the Introduction and Discussion. Furthermore, they should compare the sequences they have identified to the one in Granja’s paper and should remove all sentences from the paper relating to the fact that it is the first description of BATF3 genes in fish.

Thus, although this is a straightforward paper with no major issues, it is based almost exclusively on real time PCR analysis that provide almost no information on the role that these molecules have in fish DCs. Thus, in my opinion, the authors should have included additional studies at a cellular level to complete the paper, increasing its novelty and relevance.

Response: We edited the text according to the reviewer’s comments. We are sorry that the work published by Granja et al. was missed. As suggested, we have now commented on the findings of this work and revised the relevant text to cite this paper. However, this study does not characterise the genes per se, and simply reports a pair of primers for studying expression in the cells analysed in their study.

Minor points:

- The fish of fish sampled in the Yersinia challenge experiment should be indicated in the Materials and Methods section. In the figure legend, it is indicated that the n was 3 which is quite low.

Response: The fish number for tissue sampling in the Yersinia challenge experiment is now given in the Materials and Methods.

- At some point it is stated that VHSV is mentioned instead of IHNV (point 2.8).

Response: The error has been corrected.

- Is the leucine-zipper region of human BATF3 against which the antibody used was constructed conserved in trout BATF3? Does it recognize both forms equally? If the region is not conserved 100% the authors should demonstrate somehow that it is really recognizing trout BATF3 and only this.

Response: we evaluated the cross-activity of the BATF3 polyclonal antibody with bacterial-derived recombinant proteins of trout BATF3a and BATF3b by Western blotting. As expected, the antibody could detect both forms of trout BATF3.

- Since an important part of the transcriptional studies performed in this paper have been in RTS11, the authors should mention what is known about the role of BATF3 in mammalian monocyte-macrophage cells.

Response: Comments on this are now included in Lines 76-79. – CHECK IF THIS HAS CHANGED!
Response: The suitability of EF1a as a reference gene for qPCR has been verified in many previous studies with salmonid species. It is considered one of the most reliable house-keeping genes whose expression is hypothesised to be static in these fish. For example, Løvoll et al. (2011) performed comparative analysis on the transcript changes of several reference genes (EF1a, beta-actin, 18S and RPS20) used for qPCR analysis on gene expression in Atlantic salmon after viral infection and concluded that EF1a was the most suitable reference gene analysed. We have now provided comments on this in the paper and included the reference (Løvoll et al. 2011).

EDITORIAL COMMENTS

Although both reviewers agree on the relevane of the results, they raised several concerns that need to be addressed. Particularly, a previous study showing that BATF3 is expressed in CD8+ DC of trout must be cited and appropriately discussed. In addition, if functional studies are not included, the conclusion should be played down.
ABSTRACT

Basic leucine zipper transcription factor ATF-like (BATF) -3 is a member of the activator protein 1 (AP-1) family of transcription factors and is known to play a vital role in regulating differentiation of antigen-presenting cells in mammals. In this study, two BATF3 homologues (termed BATF3a and BATF3b) have been identified in rainbow trout (Oncorhynchus mykiss). Both genes were constitutively expressed in tissues, with particularly high levels of BATF3a in spleen, liver, pyloric caecae and head kidney. BATF3a was also more highly induced by PAMPs and cytokines in cultured cells, with type II IFN a particularly potent inducer. In rIL-4/13 pre-stimulated cells, the viral PAMPS polyI:C and R848 had the most pronounced effect on BATF3 expression. BATF3 expression could also be modulated in vivo, following infection with Yersinia ruckeri, a bacterial pathogen causing redmouth disease in salmonids, or with the rhabdovirus IHNV. The results suggest that BATF3 may be functionally conserved in regulating the differentiation and activation of immune cells in lower vertebrates and could be explored as a potential marker for comparative investigation of leucocyte lineage commitment across the vertebrate phyla.
1. The BATF3 genes were analysed in detail for the first time in fish.
2. Trout BATF3a is highly expressed in spleen, liver and pyloric caeca.
3. Trout BATF3a is highly up-regulated in monocytes/macrophages by IFNγ treatment.
4. Both BATF3a and BATF3b are induced after infection with bacterial and viral diseases.
Immune-modulation of two BATF3 paralogues in rainbow trout *Oncorhynchus mykiss*  

Jun Wang, Min Liu, Yang Wu, Sohye Yoon, Abdo Alnabulsi, Fuguo Liu, Clara Fernández-Álvarez, Tiehui Wang, Jason W. Holland, Chris J. Secombes and Jun Zou*  

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Key words: BATF3, transcription factor, leucocyte differentiation, bacterial and viral infection, rainbow trout  

Abbreviations: AP-1, activator protein 1; BATF3, basic leucine zipper transcription factor ATF-like (BATF) -3; bZIP, basic leucine zipper; CD, cluster of differentiation; DB, DNA binding domain; DC, dendritic cell; HK, head kidney; IFN, interferon; IL, interleukin; IRF, interferon regulated factor; LPS, lipopolysaccharide; LZ, leucine
zipper; PAMP, pathogen-associated molecular pattern; PHA, phytohaemagglutinin; 
polyI:C, polyinosinic:polycytidylic acid; TLR, Toll-like receptor.

**ABSTRACT**

Basic leucine zipper transcription factor ATF-like (BATF) -3 is a member of the 
avtator protein 1 (AP-1) family of transcription factors and is known to play a vital 
role in regulating differentiation of antigen-presenting cells in mammals. In this study, 
two BATF3 homologues (termed BATF3a and BATF3b) have been identified in 
rainbow trout (*Oncorhynchus mykiss*). Both genes were constitutively expressed in 
tissues, with particularly high levels of BATF3a in spleen, liver, pyloric caeca and 
head kidney. BATF3a was also more highly induced by PAMPs and cytokines in 
cultured cells, with type II IFN a particularly potent inducer. In rIL-4/13 
pre-stimulated cells, the viral PAMPS polyI:C and R848 had the most pronounced 
effect on BATF3 expression. BATF3 expression could also be modulated in vivo, 
following infection with *Yersinia ruckeri*, a bacterial pathogen causing redmouth 
disease in salmonids, or with the rhabdovirus IHNV. The results suggest that BATF3 
may be functionally conserved in regulating the differentiation and activation of 
immune cells in lower vertebrates and could be explored as a potential marker for 
comparative investigation of leucocyte lineage commitment across the vertebrate 
phyla.

**Highlights**

1. The BATF3 genes were analysed in detail for the first time in fish.
2. Trout BATF3a is highly expressed in spleen, liver and pyloric caeca.
3. Trout BATF3a is highly up-regulated in monocytes/macrophages by IFNγ 
treatment.
4. Both BATF3a and BATF3b are induced after infection with bacterial and viral 
diseases.
5. The BATF3 genes were identified for the first time in fish.
6. Trout BATF3a is highly expressed in spleen, liver and pyloric caeca.
3. Trout BATF3a is most highly up-regulated in monocytes/macrophages by IFNγ.

4. Both BATF3a and BATF3b are induced after infection with viral diseases.

1. Introduction

Basic leucine zipper transcription factor ATF-like (BATF) proteins are a group of small transcription factors belonging to the activation protein 1 (AP-1) superfamily which consist of several basic leucine zipper (bZIP) transcription factors including FOS, JUN and ATF (Landschulz et al., 1988; Murphy et al., 2013). Three BATF proteins (BATF1-3) have been characterised and all comprise an α-helical bZIP domain which can be further divided into a DNA-binding motif and a leucine zipper motif. The leucine zipper motif is known to be essential for the interaction with bZIP proteins or non-bZIP transcription factors such as interferon regulatory factors (IRFs) to regulate target genes.

The BATF3 gene has been described in mice and humans. It exists as a single copy in chromosome 1 in both species, upstream of another AP-1 family member ATF3 (Murphy et al., 2013). The genes encode a protein of 127 aa and 118 aa respectively, that share high homology (80% similarity). It lacks a signal peptide and binds to short nucleotide motifs in the promoter region of target genes. The primary sequences of human and mouse BATF3 proteins have share high homology (sharing 80% similarity). It has been shown that the BATF3 gene is expressed mainly in immune cells originating in hematopoietic organs (Williams et al., 2001), and in particular Expression of BATF3 is mainly studied in dendritic cells (DCs). For example, it can be at detectable levels in the common dendritic cell (cDC) precursors such as monocytes (which also mature develop into macrophages) and is but increases induced when DCs differentiate into fully developed conventional DCs (cDCs) (Hildner et al., 2008). In mice, BATF3 is found in both lymphoid-resident CD8α+ cDCs and non-lymphoid CD103+ cDCs which are speculated to share a common origin (Ginhoux et al., 2009; Edelson et al., 2010). T helper cells such as Th1...
and Th17 cells also express BATF3 (Hildner et al., 2008).

The central roles of BATF3 in orchestrating leucocyte lineage commitment have drawn significant attention in recent years. Emerging evidence indicates that BATF3 together with other members of the BATF family play critical roles in regulating leucocyte differentiation, especially in directing the commitment of DC precursors into specific lineages. Gene-knockout studies in mice demonstrate that BATF3 is indispensable for the development of cDCs. The Batf3−/− mice do not develop CD8α+ cDCs which are required for cytotoxic T cell immunity and antiviral defence (Hildner et al., 2008; Sun et al., 2017). In adult mice, the intestinal BATF3-dependent cDCs are required for homeostasis and antiviral T-cell immunity (Edelson, KC et al., 2010; Sun et al., 2017). Further, tissue-resident BATF3-dependent CD103+ DCs once activated can produce large amounts of interleukin (IL)-12, promoting a local Th1 response to combat *Leishmania major* infection (Martinez-Lopez et al., 2015).

However, other members of the BATF3 family may also be involved in regulation of immune responses. Recent studies indicate that the roles of BATF3 in promoting expansion of functional CD8+ cDCs to control infection of intracellular pathogens may be compensated by other members of the BATF family via the interaction of the conserved LZ domain with IRF4 or IRF8 (Tussiwand et al., 2012).

A recent study has shown that in rainbow trout (*Oncorhynchus mykiss*) skin CD8α+MHC II+ DC-like cells constitutively express BATF3. This finding is interesting and implies that the BATF3 may have conserved roles during vertebrate evolution (Granja et al., 2015). Since no further analysis of BATF3 has been undertaken to date, in this study we determined initially whether other BATF3 paralogues exist in teleost/salmonid fish, as a consequence of the 3rd or 4th whole genome duplication events seen in these species, and analysed the phylogeny of BATF3 in the context of vertebrate phyla. Two BATF3 homologues were identified in rainbow trout (*Oncorhynchus mykiss*) and their phylogeny of BATF3 was studied in the context of vertebrate phyla. Expression of the trout BATF3 paralogues was studied in vivo after bacterial and viral infection and in vitro in cultured monocytes/macrophages after stimulation with TLR ligands, a lectin and...
interferons (IFNs). The results provide a first insight into the evolution of BATF3 in lower vertebrates and will help develop potential comparative markers to study lineage commitment and differentiation between fish and higher vertebrates.

2. Materials and methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) weighing (approximately) ~100 g were maintained in 1 m diameter tanks supplied with a continuous flow of recirculating freshwater at 15 ± 1°C in the aquarium facilities in the Zoology building, University of Aberdeen. Fish were fed with commercial trout pellets (EWOS) and acclimated to aquarium conditions for at least 2 weeks before use. Fish were anaesthetised using 2-phenoxyethanol (0.05%, Sigma Aldrich) and killed by subsequent destruction of the brain prior to tissue harvest. All experiments at Aberdeen were carried out under the UK Home Office project license PPL 60/4013. For the IHNV—*infectious hematopoietic necrosis virus* (IHNV) challenge experiment, rainbow trout weighing ~3 g were obtained from the cold-water fish experiment station (Mudanjiang, China) and maintained in 120 cm × 50 cm × 60 cm tanks with aeration at 16 °C. The fish were fed daily with a dry pellet food and were also acclimated to aquarium laboratory conditions for at least 2 weeks before use. The experiment was undertaken according to the guidance of the local animal ethics committee.

2.2. RNA extraction, cDNA synthesis and gene cloning

The trout tissues and cells were collected for extraction of total RNA using TRI Reagent® (Sigma-Aldrich, UK) according to the manufacturer’s instructions. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK). The cDNA samples were kept at -20°C before use.
The human BATF3 sequence (GenBank Acc. No., NP_061134) was used as the bait sequence to undertake the BLAST (tBLASTn) analysis of the Whole-genome shotgun (WGS) database, transcriptome shotgun assembly (TSA) database and expressed sequence tags (ESTs) database, to obtain the trout BATF3 sequences. The WGS contigs were retrieved and analyzed for prediction of coding sequences using the GenScan program (Burge and Karlin, 1997). Predicted potential coding DNA sequence (CDS) were confirmed for sequence similarity by the BLASTp analysis in the non-redundant protein sequence database. Two WGS contigs (Accession Nos., CCAF010027628.1 and CCAF010060656.1) were identified to contain homologues of BATF3 genes and contained complete coding sequences (CDS) and untranslated regions (UTRs). Primers (supplementary Table 1) located in the 5’ and 3’ UTRs were designed for amplification of full length cDNA using trout head kidney cDNA as template. The PCR reaction volume was 25 μL including 2 μL of each of the primers (10 μM), 2 μL of cDNA, 5 μL of 5 × MyFi Reaction Buffer, 13 μL of PCR water and 1 μL MyFi DNA Polymerase (Bioline, UK). The PCR reaction conditions were performed using the following program: 95°C for 3 min, followed by 35 cycles at 95°C for 15 s, 62°C for 30 s, 72°C for 1-2.5 min, and a final extension at 72°C for 5 min. The purified PCR products were cloned into the pGEM®-T Easy cloning vector (Promega, UK) and transformed into RapidTrans™ TAM1 competent Escherichia coli cells (Active Motif, Belgium). The transformed cells were cultured on LB agar plates (Sigma-Aldrich, UK) with ampicillin (100 µg/mL) overnight at 37°C and colonies were screened by colony PCR using the vector specific primer M13F and a gene specific primer (supplementary Table 1.). Plasmid DNA was purified using a QIAprep® spin DNA miniprep kit (QIAGEN, UK) according to the manufacturer's instructions and the size of the inserts was verified by digestion with the restriction enzyme, EcoRI (New England Biolabs, UK). Plasmids were sequenced by Eurofins MWG Operon.

2.3. Bioinformatics analyses

The CDS regions and deduced amino acid sequences of BATF3s were analyzed using the ExPASy Translate tool (http://web.expasy.org/translate/) and the homology was analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast) against
the proteins in the National Center for Biotechnology Information (NCBI). The gene structure was predicted using the Spidey program at NCBI (http://www.ncbi.nlm.nih.gov/spidey/). Genome synteny data were obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html) for Mammalia (human and mouse), Aves (chicken), Amphibia (Xenopus tropicalis) and Teleostei.

Alignment of protein sequences between *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Chrysemys picta bellii*, *Xenopus tropicalis*, *Danio rerio*, *Oreochromis niloticus*, *Salmo salar* and *O. mykiss* was conducted using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). Protein domains were predicted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The tertiary structure of domains was predicted using CPHmodels 3.2 Server (http://www.cbs.dtu.dk/services/CPHmodels/). Domain identity/similarity was analyzed using Pair-wise sequence alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Protein sequences of selected vertebrate BATF1, BATF2 and BATF3 homologues were aligned with the ClustalW program and a phylogenetic tree was constructed using the Mega 6.0 software (Tamura et al., 2013). The neighbour-joining algorithm was used as the clustering method and the distances matrix calculated using the Poisson correction method (Saitou and Nei, 1987). The bootstrap values of tree nodes were obtained by 10,000 bootstrap repetitions using the Poisson model for amino acid substitution.

### 2.4. Tissues distribution of BATF3

Multiple tissues including brain, intestine, pyloric caeca, gill, thymus, muscle, spleen, liver and head kidney were collected from healthy rainbow trout to examine the expression of BATF3 by real-time PCR. The real-time PCR was performed using IMMOLASE (Bioline, UK) and SYBR Green fluorescent tag (Invitrogen, UK) in a LightCycler® 480 System (Roche Applied Science, UK). The primers for gene expression were designed to span exons (supplementary Table 1), to exclude the amplification of potential genomic DNA contamination.
The PCR reaction consisted of 2 μL of primers (10 pmol each), 4 mL of cDNA and 14 μL of PCR mix. The program was as follows: 10 min at 95°C for enzyme activation, 40 amplification cycles (95°C for 30 s, 60-63°C for 30 s and 72°C for 30 s), followed by 5 s at 90°C to obtain the melting curve. The serially diluted purified PCR products were used as standards to serve as reference to establish standard curves for quantification in each 96-well plate. The relative expression level of the BATF3 was calculated as arbitrary units and normalised against the expression level of rainbow trout elongation factor (EF)-1α. It has been shown previously that EF-1α was one of the suitable house-keeping genes for use in salmonid gene expression studies, as seen in studies of viral infection in Atlantic salmon where whose expression was not altered substantially during viral infection (Løvoll et al., 2011).

2.5. Expression of BATF3 in RTS-11 cells treated by TLR ligands and interferons.

The expression of BATF3 was studied in the mononuclear/macrophage -like cell line, RTS-11, from rainbow trout spleen (Brubacher, Secombes et al., 2000). The cells were maintained in Leibovitz (L-15) medium (Invitrogen, UK) containing 30% fetal calf serum (FCS; Labtech International, UK) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; P/S; Invitrogen, UK) at 20°C. Five mL of cells (approximately 1×10^6 cells) were seeded into 25 cm² flasks (Sarstedt, Germany), cultured overnight and then stimulated for 6 or 24 h with LPS (20 μg/mL; E. coli strain 055:B5; Sigma-Aldrich, UK), recombinant Yersinia ruckeri Flagellin (rFlagellin) (20 ng/mL) (Wangkahart et al., 2016), polyI:C (50 μg/mL; Sigma-Aldrich, UK), R848 (10 μg/mL; Sigma-Aldrich, UK), PHA (10 μg/mL; Sigma-Aldrich, UK), recombinant interferon 2 (rIFN2) (20 ng/mL) (Zou et al., 2007), rIFNγ (20 ng/mL) (Zou et al., 2005) or phosphate buffered saline (PBS) as control. The purity and activities of recombinant cytokines were evaluated previously (Zou et al., 2005; 2007; Gao et al., 2009; Skjesol et al., 2010; Wangkahart et al., 2016; Zou et al., 2005; 2007). Real-time PCR analysis was performed as described above.
2.6. Expression of BATF3 in primary head kidney monocytes/macrophages and rIL-4/13A cultured monocytes/macrophage cells treated with TLR ligands and/or interferons

The primary head kidney (HK) monocytes/macrophages were isolated from freshly killed rainbow trout using the method described previously by Peddie et al. (Peddie et al., 2001). Briefly, fish were anaesthetised, killed, and the anterior kidney removed aseptically and passed through a 100 µm nylon mesh using L-15 medium supplemented with P/S, heparin (10 units/mL), and 2% FCS. After centrifugation at 400 x g for 10 min at 4°C, the primary HK cells were resuspended in incomplete cell culture medium (L-15, P/S, 0.1% FCS) and washed once. The cell suspension (5 x 10^6 cells) was seeded into 25 cm² flasks containing incomplete cell culture medium (L-15, P/S, 0.1% FCS) and incubated at 20°C overnight. The unattached cells were carefully removed and complete medium (L-15, P/S, 10% FCS) was added to the flasks. The adherent cells (mostly monocytes/macrophages) were stimulated with LPS, rFlagellin, polyI:C, R848, PHA, rIFN2, rIFNγ or PBS for 24 h as described above and then harvested for real-time PCR analysis.

Archived cDNA samples from rIL-4/13A-cultured primary HK monocytes/macrophages were analysed for the expression level of BATF3 (Wang et al., 2018). Briefly, the adherent primary HK monocytes/macrophages cells were cultured in complete medium containing 200 ng/mL of rIL-4/13A. At days 1, 3 and 5, the medium was replaced with fresh complete medium containing 200 ng/mL rIL-4/13A. At day 7, the cells were stimulated with LPS, rFlagellin, polyI:C or R848. After 24 h, the cells were harvested for gene expression analysis by real-time PCR.

2.7. Expression of BATF3 in spleen, gills and intestine during Y. ruckeri infection

The expression of BATF3 was determined in archived cDNA samples of spleen, gills and intestine taken from rainbow trout after intraperitoneal injection with a
pathogenic strain (MT3072) of *Y. ruckeri* (0.5 mL/fish, $1 \times 10^6$ cfu/mL) or 0.5 mL of PBS as control (Gorgoglione-Wang et al., 2018; et al., 2016). Tissues from three fish were taken at 24 h post-challenge and analyzed by real-time PCR.

### 2.8. Expression of BATF3 in kidney during IHNV infection

Six groups of 10 healthy rainbow trout (weighing $\approx 3$ g, each group containing 10 fish) were used for the IHNV challenge experiment. Preparation of IHNV (strain HLJ-09) was described previously (Wang et al., 2016a; Wang et al., 2016b). Thirty fish were injected intraperitoneally with 50 μL of L-15 medium containing $1 \times 10^5$ pfu of IHNV. Mock-infected control groups were injected with PBS only. Head kidney was collected from infected and control fish at days 1, 3 and 5 (10 fish per group) for extraction of total RNA, using an Omega Bio-Tek extraction kit I (Omega Bio-Tek, Doraville, GA, USA) following the manufacturer’s instructions. cDNA was synthesised using oligo(dT)15 (Takara, Japan) and a Superscript Reverse Transcriptase Reagent Kit (Takara, Japan). Real-time PCR was performed using SYBR Premix EX Taq II (Takara, Japan) on the ABI 7500 real-time PCR system (Applied biosystems, Carlsbad, CA, USA) using the following conditions: 1 cycle of 30 s at 95°C, 40 cycle of 3 s at 95°C, and 30 s at 60°C. The average cycle threshold (Ct) was calculated from triplicate measurements using the instrument’s software in “auto Ct” mode (ABI 7500 system, version 2.3). Relative Ct values of three independent tests were calculated by the $2^{-\Delta\DeltaCt}$ method. EF-1α was used as an internal reference for normalization of gene expression. Infection of VHSV–IHNV was verified by examining expression of the IHNV N gene and Mx gene by real-time PCR. The primers for the IHNV N gene and Mx gene were listed in supplementary Table 1.

### 2.9. Immunohistochemical staining

A rabbit polyclonal antibody against the conserved leucine-zipper region of
human BATF3 (Merck, Cat. No. ABE1007) was used in immunohistochemical staining. To verify the cross-reactivity of this human polyclonal BATF3 antibody with trout BATF3a and BATF3b, the full length cDNA fragments were amplified and cloned into the pHISTEV vector (kindly provided by Dr Hai Deng, University of Aberdeen) at the BamHI/HindIII sites. The resultant plasmids (pHISTEV-BATF3a and pHISTEV-BATF3b) were transformed into E. coli BL21 (DE3) cells. The cells were then induced by 2 mM IPTG overnight at 37°C in a shaker (150 rpm) and 20 µL of cell culture collected for SDS-PAGE gel electrophoresis and Western blotting. The rabbit anti-human BATF3 polyclonal antibody and the secondary goat anti-rabbit IgG-peroxidase antibody (Sigma) were diluted by 1:100 (v/v) and 1:10,000 (v/v) respectively. Since the recombinant proteins have a 6-histidine tag at the N-terminus, the mouse monoclonal anti-polyhistidine-peroxidase antibody (Sigma, 1:2,000, v/v) was used to validate the recombinant trout BATF3a and BATF3b detected by the human BATF3 antibody.

The kidney tissue (100-150 mg) from healthy trout was fixed using 4% paraformaldehyde (PFA) in sterile PBS for 20 h at 4°C followed by 5 washes in sterile PBS. Tissue was incubated in the final PBS wash for 1 h at 4°C and stored at 4°C in 70% ethanol prior to further tissue processing. Tissue was embedded into paraffin wax using standard histological methods (http://www.ihcworld.com/_protocols/histology/paraffin_section.htm).

Immunohistochemistry was performed using a Dako autostainer E 172566 (Model: LV-1, Dako, UK) as described previously (Alnabulsi et al., 2017; Swan et al., 2016). The tissue sections were first dewaxed in xylene for a minimum of 10 min and rehydrated by immersion in decreasing ethanol concentrations. Then, antigen retrieval was performed by heating the tissue sections for 20 min in a microwave (800 W) while sections were fully immersed in 10 mM citrate buffer (pH 6.0). After cooling, the sections were incubated with or without (negative control) a rabbit polyclonal antibody against the conserved leucine-zipper region of human BATF3 (1:100, v/v, Merck, Cat. No. AB1007) for 60 min at room temperature. The sections were then washed twice with washing buffer (Dako), blocked incubated with blocking solution...
supplied by DAKO to block endogenous peroxidase activity for 7 min, and subsequently washed off with two buffer washes. Peroxidase-polymer labelled goat anti-mouse/rabbit secondary antibodies (Envision, Dako) was applied for 30 min at room temperature before being washed off with two buffer washes. To reveal sites of peroxidase activity, the tissue sections were treated with diaminobenzidine substrate for 7 min, followed by one distilled water wash. Finally, the slides were immersed in Surgipath Harris haematoxylin solution (Leica Biosystems) copper sulphate (WHY?) for 2 min and Harris? haematoxylin solution for 10 s to counterstain the cell nuclei, before being dehydrated in alcohol, then xylene and mounted. An antibody diluent (Dako) was used as negative control by incubating the slides with diluent instead of the primary antibody. The cell nuclei were counterstained by immersing the slides in filtered Harris haematoxylin solution before the control slides were then treated as above, dehydrated in alcohol, then xylene and mounted. Lastly, the slides were examined by light microscopy using an Olympus BX 51 light microscope (Olympus, Southend-on-Sea, Essex, UK) equipped with an Olympus C4040 camera (Olympus).

2.9. Statistical analysis

All data were analyzed using the statistical package SPSS 19.0 (SPSS Inc, Chicago, IL) software. Statistical analyses were performed using the two tailed paired Student's T-test. Data were expressed as means ± standard error (SE), with p < 0.05 considered significant.

3. Results

3.1. Cloning and sequence analysis of BATF3s genes

Two BATF3 paralogues (BATF3a and BATF3b) were obtained from the rainbow trout draft genome. One matched the primer sequences reported in Granja et al. (2015) and was called BATF3a, whilst the second was termed BATF3b. The cloned cDNA
sequences of BATF3a (GenBank Acc. No., accession number: KX826998) and BATF3b (GenBank Acc. No., accession number: KX826999) were 691bp and 594bp in length, with putative ORFs of 372 bp and 390 bp translating into proteins of 123 aa and 129 aa, respectively (supplementary Table S2, Fig. S1 and Fig. S2). Sequence comparison of the cDNA and genome sequences (GenBank Acc. Nos., CCAF010060656.1 and CCAF010027628.1) revealed that both BATF3 genes have 3 exons and 2 introns (supplementary Fig. S1 and Fig. S2).

3.2. Gene synteny analysis

Gene synteny of BATF3 genes was analyzed with neighbouring genes of Mammalia (human and mouse), Aves (chicken), Amphibia (X. tropicalis) and Teleostei. The trout BATF3a and BATF3b genes are located in two separate scaffolds (scaffold_324 and scaffold_1368), but both reside next to the ATF3 gene (Fig. 1). The tandem linkage of the BATF3 and ATF3 genes in the genome is conserved from fish to humans, suggesting that the two genes could have been duplicated from a common ancestor early in vertebrate evolution. A single copy of BATF3 is found in all of the vertebrates examined in this study except for rainbow trout and Atlantic salmon, and its gene synteny is well conserved across the vertebrate spectrum. It is common that salmonids have gene paralogues due to the 4th extra-round of genome duplication that has occurred in this lineage in addition to the teleost-specific (3rd) whole genome duplication.

3.3. Domain and phylogenetic tree analysis

BATF is a nuclear basic leucine zipper protein that belongs to the AP-1/ATF superfamily of transcription factors (Echlin et al., 2000). AP-1/ATF family members possess a typical basic leucine zipper (bZIP) DNA binding and oligomerization motif which is essential for them to form homo- or hetero-dimers with preferred binding to AP-1 or CRE (cyclic AMP-response element) sites in target DNA regions (Williams et
The bZIP domain consists of a DNA binding domain (DB), a hinge (H) region and a leucine zipper (LZ). These domains are present in all the BATF3s. The amino acid sequences of these domains are highly homologous and the six leucine residues are absolutely conserved. However, the N- and C-terminal regions of BATF3 share relatively low sequence homology. Further, a single α-helix is predicted for trout BATF3s, as in human BATF3 (Fig. 3B).

To gain a better understanding of the evolutionary relationships of rainbow trout BATF3s with known members of the vertebrate BATF family (BATF1, BATF2 and BATF3), BATF sequences from selected vertebrates, including elasmobranch (shark), ray-finned fish (medaka, pike, salmon, spotted gar, tilapia, medaka, zebrafish), lobe-finned fish (coelacanth), amphibian (frog), reptilian (turtle), avian (chicken) and mammalian (human and mouse) species, were used to construct a phylogenetic tree using the Neighbour-joining (N-J) method. The trout BATF3s branched closely with salmon BATF3s and formed a clade with other vertebrate BATF3s (bootstrap value=78%) that was separate to the BAFT1 and BATF2 clades (Fig. 4).

### 3.4. Constitutive expression analysis

The expression of trout BATF3a and BATF3b were examined in tissues of healthy fish including spleen, liver, pyloric caeca, head kidney, intestine, skin, brain, gills, brain head kidney, intestine, liver, pyloric caeca, skin, spleen, and thymus (Fig. 5). The transcript level of BATF3a was much higher than BATF3b in most tissues. The highest level of BATF3a was detected in spleen, followed by liver, pyloric caeca and head kidney.

Since relatively high transcript expression was seen in immune organs, such as spleen and kidney, the distribution of BATF3 expressing cells was studied in kidney tissue of healthy fish. It must be noted that the polyclonal antibody against the conserved leucine-zipper region of human BATF3 was used, and that it cross-reacted with both trout BATF3a and BATF3b was first confirmed by Western blotting (Fig. 6A). BATF3 expressing cells were clearly visible in the kidney inter-tubule spaces where
haematopoietic cells reside and adjacent to melan-macrophages (Fig. 6B). It must be noted that the polyclonal antibody used cross-reacted with both BATF3a and BATF3b as confirmed by Western blotting (Fig. 6).

3.5. Modulation of BATF3a and BATF3b expression in vitro

RTS-11 is a monocyte/macrophage like cell line derived from spleen. When stimulated with TLR ligands, PHA and type I and II rIFNs for 24 h, BATF3a was found to be induced by LPS, R848 and type II rIFNs but not by rFlagellin, polyI:C, PHA and type I rIFN (Fig. 7A and 7B). A moderate increase of BATF3b expression was also detected in cells treated with type II rIFN. The effects of TLR ligands were also examined at 6 h after stimulation and interestingly BATF3b was induced by rFlagellin, polyI:C and R848, with although weaker induction was seen for BATF3a with by rFlagellin and R848 (Fig. 7E). These results demonstrate that BATF3a and BATF3b are differently regulated by TLR ligands.

Primary head kidney monocytes/macrophages were next used to investigate the modulation of BATF3s expression by TLR ligands and recombinant rIFNs (Fig. 7C, D). BATF3a expression was again up-regulated by LPS, R848 and rIFNγ, the latter to a very high fold increase. However, it was also induced by the other stimulants unlike the response in RTS-11 cells. In contrast, BATF3b was induced only by flagellin, polyI:C and rIFNγ.

In our previous study, recombinant trout IL-4/13A cultured cells derived from head kidney monocytes/macrophages expressed a high level of MHCII and a moderate level of putative DC markers such as CLEC4T1/DC-SIGN and CD83, and displayed a similar morphology to mammalian DCs, with dendrites on the cell surface (Wang, Wang et al., 2016; Johansson-Wang et al., 2016; Wang et al., 2018). Archived rIL-4/13A cultured primary head kidney monocyte/macrophage cDNA samples from TLR ligand treated cells were used to investigate the expression of BATF3 (Fig. 8). Interestingly, BATF3a and BATF3b were both significantly up-regulated in rIL-4/13A cultured cells by polyI:C and R848 (viral PAMPs) stimulation. BATF3a was also
up-regulated to some extent by LPS treatment. No significant changes of BATF3b were detected in cells treated with LPS and Flagellin (bacterial PAMPs).

3.6. Modulation of BATF3a and BATF3b expression during infection

Enteric redmouth disease (ERM) is a serious septicaemic bacterial disease of salmonid fish species caused by infection with *Yersinia ruckeri* (Harun et al., 2011). A pathogenic strain (MT3072) of *Y. ruckeri* was used to infect trout by intraperitoneal injection (Wang et al., 2018). Expression of BATF3a and BATF3b was examined in systemic (spleen) and mucosal tissues (gills and intestine) tissues. A marked increase of transcripts was seen for both genes in spleen whilst they were moderately up-regulated in intestine (Fig. 9). No significant modulation of expression was detected for either gene in gills.

The expression of BATF3 genes was lastly examined in trout juveniles during infection with IHNV. IHNV is a member of the Rhabdoviridae family and infects salmonid juveniles. A recently identified IHNV strain (HLJ-09) was used to infect trout juveniles in this study. In head kidney, both BATF3a and BATF3b were up-regulated at days 3 and 5, with increases of >50-fold in the case of BATF3a at day 5 (Fig. 10). Expression of IHNV N and the antiviral gene Mx1 also increased in head kidney at days 3 and 5 post-injection (Fig. 10), verifying that infection had occurred.

4. Discussion

The BATF3 genes have not been described in non-mammalian vertebrates. In the present study, two BATF3 homologues (termed BATF3a and BATF3b) have been identified and characterized in rainbow trout (*Oncorhynchus mykiss*). BATF3a was reported previously, as constitutively expressed in CD8a+MHC II+ DC-like cells (Granja et al., 2015). We now show it is found to be more highly expressed in tissues such as spleen, liver, pyloric caeca and head kidney, and that both paralogues can be up-regulated (at least to some extent) in cultured cells by PAMPs and
cytokines, and in vivo by infection. Their potential role in fish immune responses is discussed below.

The BATF family consists of 3 members (BATF1-3) which are structurally related (Murphy et al., 2013). They contain a canonical α-helical bZIP domain and belong to the API family which includes FOS, JUN and ATF. Unlike other vertebrates which have a single copy of the BATF3 gene, trout and salmon contain two BATF3 paralogues, with the predicted proteins sharing 78.3% sequence similarity. As seen in other vertebrates, both trout BATF3a and BATF3b reside next to the ATF gene in the genome (Fig. 1). Although trout BATF3b has a slightly higher sequence homology with tetrapod BATF3 than BATF3a, all the teleost fish homologues grouped in a single clade within which the salmonid BATF3 group split into the BATF3a and BATF3b subgroups. These data support the contention that duplication of BATF3 is a salmonid-specific event likely associated with the salmonid-specific whole genome duplication (Berthelot, Brunet et al., 2014).

The expression of BATF3 is limited to leukocytes in humans and mice, and increases during cDC development. The spleen is a major secondary lymphoid organ in fish, where leukocytes (e.g. antigen presenting cells, T and B cells) interact with each other. Therefore, it is not surprising that a high level of trout BATF3a transcripts was detected in this tissue (Fig. 5). Trout BATF3a was also highly expressed in pyloric caeca. Recent studies have shown that pyloric caeca is an important organ involved in mucosal immunity and harbors a high density of B and T cells amongst others (Ballesteros et al., 2013). That the BATF3 paralogues are constitutively expressed in immune tissues suggests they may be involved in maintenance of homeostasis of the immune system in fish.

Expression modulation of trout BATF3a and BATF3b was examined in primary monocytes/macrophages isolated from head kidney and a spleen monocyte/macrophage cell line (RTS-11). In the HK monocytes/macrophages, BATF3a could be induced by all the stimuli used. Notably, stimulation with IFNγ resulted in the largest increase of BATF3a expression (55-fold increase). In contrast to the HK monocytes/macrophages, only small changes in BATF3a and BAT3b
expression were seen largely unaltered in the RTS-11 cells, where relatively small increases were seen after stimulation with LPS, Flagellin, poly I:C, R848 and rIFNγ, with modulation of BATF3b mainly seen at the earlier time (6 h) post-stimulation. It should be noted that the zinc finger transcription factors ZBTB46 and DC-SCRIPT/ZNF366 known to be involved in activation of antigen presenting cells, were down-regulated in primary HK monocytes/macrophages by rIFNγ, highlighting the central roles of rIFNγ in mediating antigen presentation in fish (Zou et al., 2005; Wang et al., 2018; Wiegertjes et al., 2016; Zou et al., 2005).

IL-4 in combination with granulocyte macrophage colony stimulating factor (GM-CSF) is commonly used for in vitro culture of dendritic cells in humans and mice (Dauer et al., 2003). Two IL-4/13 homologues (IL-4/13A and IL-4/13B) have been identified in rainbow trout but GM-CSF is absent in teleost fish (Zou and Secombes, 2011; Wang et al., 2016; Zou and Secombes, 2011). Trout rIL-4/13A can enhance HK monocyte/macrophage differentiation into CLEC4T+ cells (Johansson et al., 2016). These cells express a remarkably high level of MHC II molecules and moderate levels of the macrophage colony stimulating factor receptor (MCSFR) and CD83, and display a distinct morphology when stimulated with bacterial and viral PAMPs in association with inducible expression of ZBTB46 (Wang et al., 2018). In such cells, cultured with rIL-4/13A for 7 days, the transcript levels of BATF3a and BATF3b remained largely unchanged (7-10 x 10^{-4} of BATF3a relative to EF-1α, 1-3 x 10^{-4} of BATF3b relative to EF-1α) (Figs. 5 and 8), but further stimulation, in particular with viral PAMPs, enhanced expression several fold.

The in vivo studies demonstrated that the BATF3 paralogues are also modulated during infection, in this case by bacterial and viral diseases. Relatively high increases in transcript level were seen in the target organs; spleen in the case of Yersiniosis and kidney in the case of IHNV. The kinetics of induction were also studied during the case of IHNV, where maximal increases were seen several days post-infection. Taken together, the present findings suggest that BATF3 genes may have both homeostatic and inducible functions within the immune system of fish, potentially in the context of DC differentiation and activation. The characterization of BATF genes...
in fish provides useful data for further characterization of the role(s) of BATF3 in regulating leucocyte differentiation in early vertebrates.

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Figure legend

Fig. 1. Comparative analysis of gene synteny of BATF3. The rainbow trout WGS scaffolds were obtained from NCBI, and the gene synteny of other vertebrates was obtained from the Ensemble Genome Browser (http://sep2015.archive.ensembl.org/index.html). The arrows indicate the transcriptional direction. The homologous genes are shown with the same colour.

Fig. 2. Amino acid sequence alignment (A), and identity/similarity analysis (B) of BATF3 in Homo sapiens, Mus musculus, Gallus gallus, Chrysemys picta bellii, Xenopus tropicalis, Danio rerio, Oreochromis niloticus, Salmo salar and Oncorhynchus mykiss. Sequences were aligned using the ClustalW server (http://clustalw.ddbj.nig.ac.jp/). Sequence identity/similarity was analysed using the Pair-wise sequence alignment. Identity (*), strong similarity (:) and weak similarity (.) are indicated below the alignment. The structural domains of BATF3 were predicted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The core domain is boxed, with DNA binding domain (DB), hinge (H) and leucine zipper (LZ) regions indicated above the alignment. The conserved leucines are highlighted.

Fig. 3. Comparative analysis of BATF3 protein structure (A), tertiary structure (B) and identity/similarity of structural domains (C) between human (hu) and rainbow trout (rt). A, the structural domains of BATF3 molecules were predicted using the
The bZIP domain was constituted of a DNA binding domain, hinge region and leucine zipper region. B, the tertiary structure of bZIP domains was predicted using CPHmodels 3.2 Server (http://www.cbs.dtu.dk/services/CPHmodels/). C, domain identity/similarity was analysed using the Pair-wise sequence alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Fig. 4. Phylogenetic tree analysis of BATF1-3. The phylogenetic tree was drawn using the Mega 6.0 software. The neighbour-joining algorithm was used as the clustering method and the distances matrix computed using the Poisson correction method. The tree was supported by 10,000 bootstrap repetitions using the Poisson model for amino acid substitution. The bootstrap values are indicated as percentages.

Fig. 5. Tissue distribution of rainbow trout BATF3. The expression levels of BATF3a and BATF3b were determined by real-time PCR and normalized to the expression level of a reference gene EF-1α. The results represent the means ± SE of five fish.

Fig. 6. Western blotting (A) and immunohistochemical staining (B). Twenty µL of an IPTG-induced overnight culture of E. coli BL21 (DE3) cells transformed with pHISTEV-BATF3a (Lane 1) or pHISTEV-BATF3b (Lane 2) was analysed by Western blotting using a polyclonal antibody against the conserved leucine zipper region of human BATF3 (1:100, v/v) followed by a goat anti-rabbit IgG-peroxidase antibody (1:10,000 v/v) or the mouse monoclonal anti-polyhistidine-peroxidase antibody (Sigma, 1:2000, v/v). For immunohistochemistry, the kidney tissue (100-150 mg) of healthy trout was fixed using 4% paraformaldehyde (PFA) in sterile PBS and embedded into paraffin wax using standard histological methods. The slides were incubated with (right A) or without (left B) the rabbit polyclonal antibody against the conserved leucine zipper region of human BATF3 (1:100, v/v), and the cell nuclei were counterstained with Harris haematoxylin. Arrows indicate the positively-stained cells. Bar=50 µM.

Fig. 7. Modulation of expression of BATF3 in trout RTS-11 cells (A, B, E) and primary HK monocytes/macrophages (C, D). The cells were stimulated for 6 h (E) or
24 h (A-D-B) with LPS (20 μg/mL), rFlagellin (20 ng/mL), polyI:C (50 μg/mL), R848 (10 μg/mL), PHA (10 μg/mL), rIFN2 (type I) (20 ng/mL), rIFNγ (20 ng/mL) or PBS (control). The gene expression levels were determined by real-time PCR and normalized to that of EF-1α. The fold changes were calculated by comparing the average expression level of each treatment group with that of the respective control group. Bars indicate means ± SE of gene expression levels of cells from three flasks for RTS-11 cells (n=3) or four fish for the primary HK monocytes/macrophages (n=4).

* = p ≤ 0.05, ** = p ≤ 0.01.

Fig. 8. Expression modulation of BATF3 in trout HK monocytes/macrophages cultured with rIL-4/13A. The primary adherent HK leucocytes were isolated and cultured in the presence of 200 ng/mL rIL-4/13A for 7 days. The cells were then stimulated for 24 h with LPS (20 μg/mL), rFlagellin (20 ng/mL), polyI:C (50 μg/mL), R848 (10 μg/mL), or PBS (control). The gene expression levels were determined by real-time PCR and normalized to that of EF-1α. The fold changes were calculated by comparing the average expression level of each treatment group with that of the respective control group. Bars indicate means ± SE of gene expression levels of cells from four flasks. * = p ≤ 0.05, ** = p ≤ 0.01. A, expression of BATF3a and BATF3b in HK monocytes/macrophages cultured with rIL-4/13A for 7 days; B, fold changes of expression of BATF3a and BATF3b after stimulation.

Fig. 9. Expression of BATF3 in rainbow trout after infection of Y. ruckeri. Fish were challenged by intraperitoneal injection with a pathogenic strain (MT3072) of Y. ruckeri (5 × 10^5 cfu per fish) or PBS. Gill, spleen and intestine were collected at 24 h post-challenge and real-time PCR was performed to determine BATF3 expression. The expression levels of BATF3a and BATF3b were normalized to that of EF-1α and fold changes calculated by comparing the expression level of challenged fish with that of the respective control fish (defined as 1). Bars indicate means ± SE of tissues from three fish. * = p ≤ 0.05.

Fig. 10. Expression of BATF3, Mx1 and IHNV N in rainbow trout juveniles (~3 g) after infection with IHNV. The fish were injected intraperitoneally with 50 μL of L-15 medium containing 1 x 10^5 pfu of IHNV (strain HLJ-09). At days 1, 3 and 5, head
kidney was collected for analysis of gene expression. Bars represent the fold change of expression level compared to that of uninfected control fish. Data shown are the means ±SE of 10 fish. * = p < 0.01 compared to day 1, # = p < 0.01 compared to day 3.

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Immune-modulation of two BATF3 paralogues in rainbow trout *Oncorhynchus mykiss*

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Key words: BATF3, transcription factor, leucocyte differentiation, bacterial and viral infection, rainbow trout

Abbreviations: AP-1, activator protein 1; BATF3, basic leucine zipper transcription factor ATF-like (BATF) -3; bZIP, basic leucine zipper; CD, cluster of differentiation; DB, DNA binding domain; DC, dendritic cell; HK, head kidney; IFN, interferon; IL, interleukin; IRF, interferon regulated factor; LPS, lipopolysaccharide; LZ, leucine
ABSTRACT

Basic leucine zipper transcription factor ATF-like (BATF) -3 is a member of the activator protein 1 (AP-1) family of transcription factors and is known to play a vital role in regulating differentiation of antigen-presenting cells in mammals. In this study, two BATF3 homologues (termed BATF3a and BATF3b) have been identified in rainbow trout (Oncorhynchus mykiss). Both genes were constitutively expressed in tissues, with particularly high levels of BATF3a in spleen, liver, pyloric caecae and head kidney. BATF3a was also more highly induced by PAMPs and cytokines in cultured cells, with type II IFN a particularly potent inducer. In rIL-4/13 pre-stimulated cells, the viral PAMPS polyI:C and R848 had the most pronounced effect on BATF3 expression. BATF3a expression could also be modulated in vivo, following infection with Yersinia ruckeri, a bacterial pathogen causing redmouth disease in salmonids, or with the rhabdovirus IHNV. The results suggest that BATF3 may be functionally conserved in regulating the differentiation and activation of immune cells in lower vertebrates and could be explored as a potential marker for comparative investigation of leucocyte lineage commitment across the vertebrate phyla.

Highlights

1. The BATF3 genes were analysed in detail for the first time in fish.
2. Trout BATF3a is highly expressed in spleen, liver and pyloric caeca.
3. Trout BATF3a is highly up-regulated in monocytes/macrophages by IFNγ treatment.
4. Both BATF3a and BATF3b are induced after infection with bacterial and viral diseases.

1. The BATF3 genes were identified for the first time in fish.
2. Trout BATF3a is highly expressed in spleen, liver and pyloric caeca.
1. Introduction

Basic leucine zipper transcription factor ATF-like (BATF) proteins are a group of small transcription factors belonging to the activation protein 1 (AP-1) superfamily which consist of several basic leucine zipper (bZIP) transcription factors including FOS, JUN and ATF (Landschulz et al., 1988; Murphy et al., 2013). Three BATF proteins (BATF1-3) have been characterised and all comprise an α-helical bZIP domain which can be further divided into a DNA-binding motif and a leucine zipper motif. The leucine zipper motif is known to be essential for the interaction with bZIP proteins or non-bZIP transcription factors such as interferon regulatory factors (IRFs) to regulate target genes.

The BATF3 gene has been described in mice and humans. It exists as a single copy in chromosome 1 in both species, upstream of another AP-1 family member ATF3 (Murphy et al., 2013). The genes encode a protein of 127 aa and 118 aa respectively, that share high homology (80% similarity). It lacks a signal peptide and binds to short nucleotide motifs in the promoter region of target genes. The primary sequences of human and mouse BATF3 proteins have share high homology (sharing 80% similarity). It has been shown that the BATF3 gene is expressed mainly in immune cells originating in hematopoietic organs (Williams et al., 2001), and in particular, Expression of BATF3 is mainly studied in dendritic cells (DCs). For example, it can be detected at detectable levels in the common dendritic cell (DC) precursors such as monocytes (which also mature into macrophages) and increases when DCs differentiate into fully developed conventional DCs (cDCs) (Hildner et al., 2008). In mice, BATF3 is found in both lymphoid-resident CD8α+ cDCs and non-lymphoid CD103+ cDCs which are speculated to share a common origin (Ginhoux et al., 2009; Edelson et al., 2010). T helper cells such as Th1

3. Trout BATF3a is most highly up-regulated in monocytes/macrophages by IFNγ.

4. Both BATF3a and BATF3b are induced after infection with bacterial and viral diseases.
and Th17 cells also express BATF3 (Hildner et al., 2008).

The central roles of BATF3 in orchestrating leucocyte lineage commitment have drawn significant attention in recent years. Emerging evidence indicates that BATF3 together with other members of the BATF family play critical roles in regulating leucocyte differentiation, especially in directing the commitment of DC precursors into specific lineages. Gene-knockout studies in mice demonstrate that BATF3 is indispensable for the development of cDCs. The Batf3−/− mice do not develop CD8α+ cDCs which are required for cytotoxic T cell immunity and antiviral defence (Hildner et al., 2008; Sun et al., 2017). In adult mice, the intestinal BATF3-dependent cDCs are required for homeostasis and antiviral T-cell immunity (Edelson-KC et al., 2010; Sun et al., 2017). Further, tissue-resident BATF3-dependent CD103+ DCs once activated can produce large amounts of interleukin (IL)-12, promoting a local Th1 response to combat Leishmania major infection (Martinez-Lopez et al., 2015).

However, other members of the BATF3 family may also be involved in regulation of immune responses. Recent studies indicate that the roles of BATF3 in promoting expansion of functional CD8+ cDCs to control infection of intracellular pathogens may be compensated by other members of the BATF family via the interaction of the conserved LZ domain with IRF4 or IRF8 (Tussiwand et al., 2012).

A recent study has shown that in rainbow trout (Oncorhynchus mykiss) skin CD8α+MHC II+ DC-like cells constitutively express BATF3. This finding is interesting and implies that the BATF3 may have conserved roles during vertebrate evolution (Granja et al., 2015). Since no further analysis of BATF3 has been undertaken to date, in this study we determined initially whether other BATF3 paralogues exist in teleost/salmonid fish, as a consequence of the 3rd or 4th whole genome duplication events seen in these species, and analysed the phylogeny of BATF3 in the context of vertebrate phyla. Two BATF3 homologues were identified in rainbow trout (Oncorhynchus mykiss) and their phylogeny of BATF3 was studied in the context of vertebrate phyla. Expression of the trout BATF3 paralogues was studied in vivo after bacterial and viral infection and in vitro in cultured monocytes/macrophages after stimulation with TLR ligands, a lectin and...
interferons (IFNs). The results provide a first insight into the evolution of BATF3 in lower vertebrates and will help develop potential comparative markers to study leucocyte lineage commitment and differentiation between fish and higher vertebrates.

2. Materials and methods

2.1. Fish

Rainbow trout (Oncorhynchus mykiss) weighing approximately ~100 g were maintained in 1 m diameter tanks supplied with a continuous flow of recirculating freshwater at 15 ± 1°C in the aquarium facilities in the Zoology building, University of Aberdeen. Fish were fed with commercial trout pellets (EWOS) and acclimated to aquarium conditions for at least 2 weeks before use. Fish were anaesthetised using 2-phenoxyethanol (0.05%, Sigma Aldrich) and killed by subsequent destruction of the brain prior to tissue harvest. All experiments at Aberdeen were carried out under the UK Home Office project license PPL 60/4013. For the IHNV—(infectious hematopoietic necrosis virus) challenge experiment, rainbow trout weighing ~3 g were obtained from the cold-water fish experiment station (Mudanjiang, China) and maintained in 120 cm × 50 cm × 60 cm tanks with aeration at 16°C. The fish were fed daily with a dry pellet food and were also acclimated to aquarium laboratory conditions for at least 2 weeks before use. The experiment was undertaken according to the guidance of the local animal ethics committee.

2.2. RNA extraction, cDNA synthesis and gene cloning

The trout tissues and cells were collected for extraction of total RNA using TRI Reagent® (Sigma-Aldrich, UK) according to the manufacturer’s instructions. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK). The cDNA samples were kept at -20°C before use.
The human BATF3 sequence (GenBank Acc. No., NP_061134) was used as the bait sequence to undertake the BLAST (tBLASTn) analysis of the Whole-genome shotgun (WGS) database, transcriptome shotgun assembly (TSA) database and expressed sequence tags (ESTs) database, to obtain the trout BATF3 sequences. The WGS contigs were retrieved and analyzed for prediction of coding sequences using the GenScan program (Burge and Karlin, 1997). Predicted potential coding DNA sequence (CDS) were confirmed for sequence similarity by the BLASTp analysis in the non-redundant protein sequence database. Two WGS contigs (Accession Nos., CCAF010027628.1 and CCAF010060656.1) were identified to contain homologues of BATF3 genes and contained complete coding sequences (CDS) and untranslated regions (UTRs). Primers (supplementary Table 1) located in the 5’ and 3’ UTRs were designed for amplification of full length cDNA using trout head kidney cDNA as template. The PCR reaction volume was 25 μL including 2 μL of each of the primers (10 μM), 2 μL of cDNA, 5 μL of 5 × MyFi Reaction Buffer, 13 μL of PCR water and 1 μL MyFi DNA Polymerase (Bioline, UK). The PCR reaction conditions were performed using the following program: 95°C for 3 min, followed by 35 cycles at 95°C for 15 s, 62°C for 30 s, 72°C for 1-2.5 min, and a final extension at 72°C for 5 min. The purified PCR products were cloned into the pGEM®-T Easy cloning vector (Promega, UK) and transformed into RapidTrans™ TAM1 competent Escherichia coli cells (Active Motif, Belgium). The transformed cells were cultured on LB agar plates (Sigma-Aldrich, UK) with ampicillin (100 μg/mL) overnight at 37°C and colonies were screened by colony PCR using the vector specific primer M13F and a gene specific primer (supplementary Table 1.). Plasmid DNA was purified using a QIAPrep® spin DNA miniprep kit (QIAGEN, UK) according to the manufacturer's instructions and the size of the inserts was verified by digestion with the restriction enzyme, EcoRI (New England Biolabs, UK). Plasmids were sequenced by Eurofins MWG Operon.

2.3. Bioinformatics analyses

The CDS regions and deduced amino acid sequences of BATF3s were analyzed using the ExPASy Translate tool (http://web.expasy.org/translate/) and the homology was analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast) against
the proteins in the National Center for Biotechnology Information (NCBI). The gene structure was predicted using the Spidey program at NCBI (http://www.ncbi.nlm.nih.gov/spidey/). Genome synteny data were obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html) for Mammalia (human and mouse), Aves (chicken), Amphibia (Xenopus tropicalis) and Teleostei. Alignment of protein sequences between Homo sapiens, Mus musculus, Gallus gallus, Chrysemys picta bellii, Xenopus tropicalis, Danio rerio, Oreochromis niloticus, Salmo salar and O. mykiss was conducted using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). Protein domains were predicted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The tertiary structure of domains was predicted using CPHmodels 3.2 Server (http://www.cbs.dtu.dk/services/CPHmodels/). Domain identity/similarity was analyzed using Pair-wise sequence alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Protein sequences of selected vertebrate BATF1, BATF2 and BATF3 homologues were aligned with the ClustalW program and a phylogenetic tree was constructed using the Mega 6.0 software (Tamura et al., 2013). The neighbour-joining algorithm was used as the clustering method and the distances matrix calculated using the Poisson correction method (Saitou and Nei, 1987). The bootstrap values of tree nodes were obtained by 10,000 bootstrap repetitions using the Poisson model for amino acid substitution.

2.4. Tissues distribution of BATF3.

Multiple tissues including brain, intestine, pyloric caeca, gill, thymus, muscle, spleen, liver and head kidney were collected from healthy rainbow trout for examining the expression of BATF3 by real-time PCR. The real-time PCR was performed using IMMOLASE (Bioline, UK) and SYBR Green fluorescent tag (Invitrogen, UK) in a LightCycler® 480 System (Roche Applied Science, UK). The primers for gene expression were designed to span exons (supplementary Table 1), to exclude the amplification of potential genomic DNA contamination during RNA isolation.
preparation. The PCR reaction consisted of 2 μL of primers (10 pmol each), 4 mL of
cDNA and 14 μL of PCR mix. The program was as follows: 10 min at 95.0°C for
enzyme activation, 40 amplification cycles (95.0°C for 30 s, 60-63.0°C for 30 s and 72.0°C
for 30 s), followed by 5 s at 90.0°C to obtain the melting curve. The serially diluted
purified PCR products were used as standards to serve as reference to establish
standard curves for quantification in each 96-well plate. The relative expression level
of the BATF3 was calculated as arbitrary units and normalised against the expression
level of rainbow trout elongation factor (EF)-1α. It has been shown previously that EF-1α was one of the suitable house-keeping genes for use in salmonid
gene expression studies, as seen in studies of viral infection in Atlantic salmon
where whose expression was not altered substantially during viral infection (Løvoll et
al., 2011).

2.5. Expression of BATF3 in RTS-11 cells treated by TLR ligands and interferons.
The expression of BATF3 was studied in the mononuclear/macrophage-like cell
line, RTS-11, from rainbow trout spleen (Brubacher, Secombes et al., 2000). The cells
were maintained in Leibovitz (L-15) medium (Invitrogen, UK) containing 30% fetal
calf serum (FCS; Labtech International, UK) and antibiotics (100 U/mL penicillin and
100 mg/mL streptomycin; P/S; Invitrogen, UK) at 20–23°C. Five mL of cells
(approximately 1 × 10^5 cells) were seeded into 25 cm² flasks (Sarstedt, Germany),
cultured overnight and then stimulated for 6 or 24 h with LPS (20 μg/mL; E. coli
strain 055:B5; Sigma-Aldrich, UK), recombinant Yersinia ruckeri Flagellin (rFlagellin)
(20 ng/mL) (Wangkahart et al., 2016), polyI:C (50 μg/mL; Sigma-Aldrich, UK), R848
(10 μg/mL; Sigma-Aldrich, UK), PHA (10 μg/mL; Sigma-Aldrich, UK), recombinant
interferon 2 (rIFN2) (20 ng/mL) (Zou et al., 2007), rIFNγ (20 ng/mL) (Zou; et al.,
2005) or phosphate buffered saline (PBS) as control. The purity and activities of
recombinant cytokines were evaluated previously (Zou et al., 2005; 2007; Gao et al.,
2009; Skjesol et al., 2010; Wangkahart et al., 2016; Zou, et al., 2005; 2007). Real-time
PCR analysis was performed as described above.
2.6. Expression of BATF3 in primary head kidney monocytes/macrophages and rIL-4/13A cultured monocytes/macrophages cells treated with TLR ligands and/or interferons

The primary head kidney (HK) monocytes/macrophages were isolated from freshly killed rainbow trout using the method described previously by Peddie et al. (Peddie et al., 2001). Briefly, fish were anaesthetised, killed, and the anterior kidney removed aseptically and passed through a 100 μm nylon mesh using L-15 medium supplemented with P/S, heparin (10 units/mL), and 2% FCS. After centrifugation at 400 x g for 10 min at 4°C, the primary HK cells were resuspended in incomplete cell culture medium (L-15, P/S, 0.1% FCS) and washed once. The cell suspension (5 × 10⁶ cells) was seeded into 25 cm² flasks containing incomplete cell culture medium (L-15, P/S, 0.1% FCS) and incubated at 20°C overnight. The unattached cells were carefully removed and complete medium (L-15, P/S, 10% FCS) was added to the flasks. The adherent cells (mostly monocytes/macrophages) were stimulated with LPS, rFlagellin, polyI:C, R848, PHA, rIFN2, rIFNγ or PBS for 24 h as described above and then harvested for real-time PCR analysis.

Archived cDNA samples from rIL-4/13A-cultured primary HK monocytes/macrophages were analysed for the expression level of BATF3 (Wang et al., 2018). Briefly, the adherent primary HK monocytes/macrophages were cultured in complete medium containing 200 ng/mL of rIL-4/13A. At days 1, 3 and 5, the medium was replaced with fresh complete medium containing 200 ng/mL rIL-4/13A. At day 7, the cells were stimulated with LPS, rFlagellin, polyI:C or R848. After 24 h, the cells were harvested for gene expression analysis by real-time PCR.

2.7. Expression of BATF3 in spleen, gills and intestine during Y. ruckeri infection

The expression of BATF3 was determined in archived cDNA samples of spleen, gills and intestine taken from rainbow trout after intraperitoneal injection with a
pathogenic strain (MT3072) of *Y. ruckeri* (0.5 mL/fish, 1 × 10^6 cfu/mL) or 0.5 mL of PBS as control (Gorgoglione et al., 2018; BUT THIS PAPER IS BROWN TROUT!). Tissues from three fish were taken at 24 h post-challenge and analyzed by real-time PCR.

2.8. Expression of BATF3 in kidney during IHNV infection

Six groups of 10 healthy rainbow trout (weighing ~3 g, each group containing 10 fish) were used for the IHNV challenge experiment. Preparation of IHNV (strain HLJ-09) was described previously (Wang et al., 2016a; Wang et al., 2016b).

Thirty fish were injected intraperitoneally with 50 μL of L-15 medium containing 1 × 10^5 pfu of IHNV. Mock-infected control groups were injected with PBS only. Head kidney was collected from infected and control fish at days 1, 3 and 5 (10 fish per group) for extraction of total RNA, using an Omega Bio-Tek extraction kit I (Omega Bio-Tek, Doraville, GA, USA) following the manufacturer’s instructions. cDNA was synthesised using oligo(dT)15 (Takara, Japan) and a Superscript Reverse Transcriptase Reagent Kit (Takara, Japan). Real-time PCR was performed using SYBR Premix EX Taq II (Takara, Japan) on the ABI 7500 real-time PCR system (Applied biosystems, Carlsbad, CA, USA) using the following conditions: 1 cycle of 30 s at 95℃, 40 cycle of 3 s at 95℃, and 30 s at 60℃. The average cycle threshold (Ct) was calculated from triplicate measurements using the instrument’s software in “auto Ct” mode (ABI 7500 system, version 2.3). Relative Ct values of three independent tests were calculated by the 2^-ΔΔCt method. EF-1α was used as an internal reference for normalization of gene expression. Infection of VHSV–IHNV was verified by examining expression of the IHNV N gene and Mx gene by real-time PCR. The primers for the IHNV N gene and Mx gene were listed in supplementary Table 1.

2.9. Immunohistochemical staining

A rabbit polyclonal antibody against the conserved leucine-zipper region of
human BATF3 (Merck, Cat. No. ABE1007) was used in immunohistochemical staining. To verify the cross-reactivity of this human polyclonal BATF3 antibody with trout BATF3a and BATF3b, the full length cDNA fragments were amplified and cloned into the pHISTEV vector (kindly provided by Dr Hai Deng, University of Aberdeen) at the BamHI/HindIII sites. The resultant plasmids (pHISTEV-BATF3a and pHISTEV-BATF3b) were transformed into E. coli BL21 (DE3) cells. The cells were then induced by 2 mM IPTG overnight at 37°C in a shaker (150 rpm) and 20 µL of cell culture collected for SDS-PAGE gel electrophoresis and Western blotting. The rabbit anti human BATF3 polyclonal antibody and the secondary goat anti-rabbit IgG-peroxidase antibody (Sigma) were diluted by 1:100 (v/v) and 1:10,000 (v/v) respectively. Since the recombinant proteins have a 6-histidine tag at the N-terminus, the mouse monoclonal anti-polyhistidine-peroxidase antibody (Sigma, 1:2,000, v/v) was used to validate the recombinant trout BATF3a and BATF3b detected by the human BATF3 antibody.

Next, the kidney tissue (100-150 mg) from a healthy trout was fixed using 4% paraformaldehyde (PFA) in sterile PBS for 20 h at 4°C followed by 5 washes in sterile PBS. Tissue was incubated in the final PBS wash for 1 h at 4°C and stored at 4°C in 70% ethanol prior to further tissue processing. Tissue was embedded into paraffin wax using standard histological methods (http://www.ihcworld.com/_protocols/histology/paraffin_section.htm).

Immunohistochemistry was performed using a Dako autostainer E 172566 (Model: LV-1, Dako, UK) as described previously (Alnabulsi et al., 2017; Swan et al., 2016). The tissue sections were first dewaxed in xylene for a minimum of 10 min and rehydrated by immersing in decreasing ethanol concentrations. Then, antigen retrieval was performed by heating the tissue sections for 20 min in a microwave (800 W) while sections were fully immersed in 10 mM citrate buffer (pH 6.0). After cooling, the sections were incubated with or without (negative control) a rabbit polyclonal antibody against the conserved leucine-zipper region of human BATF3 (1:100, v/v; Merck, Cat. No. AB1007) for 60 min at room temperature. The sections were then washed twice with washing buffer (Dako), blocked-incubated with blocking solution...
supplied by DAKO to block endogenous peroxidase activity for 7 min, and subsequently washed off with two buffer washes. Peroxidase-polymer labelled goat anti-mouse/rabbit secondary antibodies (Envision, Dako) was applied for 30 min at room temperature before being washed off with two buffer washes. To reveal sites of peroxidase activity, the tissue sections were treated with diaminobenzidine substrate for 7 min, followed by one distilled water wash. Finally, the slides were immersed in Surgipath Harris haematoxylin solution (Leica Biosystems) copper sulphate (WHY?) for 2 min and Harris? haematoxylin solution for 10 s to counterstain the cell nuclei, before being dehydrated in alcohol, then xylene and mounted. An antibody diluent (Dako) was used as negative control by incubating the slides with diluent instead of the primary antibody. The cell nuclei were counterstained by immersing the slides in filtered Harris haematoxylin solution before the control slides were then treated as above dehydrated in alcohol, then xylene and mounted. Lastly, the slides were examined by light microscopy using an Olympus BX 51 light microscope (Olympus, Southend-on-Sea, Essex, UK) equipped with an Olympus C4040 camera (Olympus).

2.9. Statistical analysis

All data were analyzed using the statistical package SPSS 19.0 (SPSS Inc, Chicago, IL) software. Statistical analyses were performed using the two tailed paired Student’s T-test. Data were expressed as means ± standard error (SE), with p < 0.05 considered significant.

3. Results

3.1. Cloning and sequence analysis of BATF3 genes

Two BATF3 paralogues (BATF3a and BATF3b) were obtained from the rainbow trout draft genome. One matched the primer sequences reported in Granja et al. (2015) and was called BATF3a, whilst the second was termed BATF3b. The cloned cDNA
sequences of BATF3a (GenBank Acc. No., accession number: KX826998) and
BATF3b (GenBank Acc. No., accession number: KX826999) were 691bp and 594bp
in length, with putative ORFs of 372 bp and 390 bp translating into proteins of 123 aa
and 129 aa, respectively (supplementary Table S2, Fig. S1 and Fig. S2). Sequence
comparison of the cDNA and genome sequences (GenBank Acc. Nos.,
CCAF010060656.1 and CCAF010027628.1) revealed that both BATF3 genes have 3
exons and 2 introns (supplementary Fig. S1 and Fig. S2).

3.2. Gene synteny analysis

Gene synteny of BATF3 genes was analyzed with neighbouring genes of
Mammalia (human and mouse), Aves (chicken), Amphibia (X. tropicalis) and
Teleostei. The trout BATF3a and BATF3b genes are located in two separate scaffolds
(scaffold_324 and scaffold_1368), but both reside next to the ATF3 gene (Fig. 1). The
tandem linkage of the BATF3 and ATF3 genes in the genome is conserved from fish
to humans, suggesting that the two genes could have been duplicated from a common
ancestor early in vertebrate evolution. A single copy of BATF3 is found in all of the
vertebrates examined in this study except for rainbow trout and Atlantic salmon, and
its gene synteny is well conserved across the vertebrate spectrum. It is common that
salmonids have gene paralogues due to the 4th round of genome duplication that
has occurred in this lineage in addition to the teleost-specific (3rd) whole genome
duplication.

3.3. Domain and phylogenetic tree analysis

BATF is a nuclear basic leucine zipper protein that belongs to the AP-1/ATF
superfamily of transcription factors (Echlin et al., 2000). AP-1/ATF family members
possess a typical basic leucine zipper (bZIP) DNA binding and oligomerization motif
which is essential for them to form homo- or hetero-dimers with preferred binding to
AP-1 or CRE (cyclic AMP-response element) sites in target DNA regions (Williams et
The bZIP domain consists of a DNA binding domain (DB), a hinge (H) region and a leucine zipper (LZ). These domains are present in all the BATF3s. The amino acid sequences of these domains are highly homologous and the six leucine residues are absolutely conserved. However, the N- and C-terminal regions of BATF3 share relatively low sequence homology. Further, a single α-helix is predicted for trout BATF3s, as in human BATF3 (Fig. 3B).

To gain a better understanding of the evolutionary relationships of rainbow trout BATF3s with known members of the vertebrate BATF family (BATF1, BATF2 and BATF3), BATF sequences from selected vertebrates, including elasmobranch (shark), ray-finned fish (medaka, pike, salmon, spotted gar, tilapia, medaka, zebrafish), lobe-finned fish (coelacanth), amphibian (frog), reptilian (turtle), avian (chicken) and mammalian (human and mouse) species, were used to construct a phylogenetic tree using the Neighbour-joining (N-J) method. The trout BATF3s branched closely with salmon BATF3s and formed a clade with other vertebrate BATF3s (bootstrap value=78%) that was separate to the BAFT1 and BATF2 clades (Fig. 4).

3.4. Constitutive expression analysis

The expression of trout BATF3a and BATF3b were examined in tissues of healthy fish including spleen, liver, pyloric caeca, head kidney, intestine, skin, brain, gills, brain, head kidney, intestine, liver, pyloric caeca, skin, spleen, and thymus (Fig. 5). The transcript level of BATF3a was much higher than BATF3b in most tissues. The highest level of BATF3a was detected in spleen, followed by liver, pyloric caeca and head kidney.

Since relatively high transcript expression was seen in immune organs, such as spleen and kidney, the distribution of BATF3 expressing cells was studied in kidney tissue of healthy fish. A polyclonal antibody against the conserved leucine-zipper region of human BATF3 was used, and it was first confirmed by Western blotting (Fig. 6A). BATF3 expressing cells were clearly visible in the kidney inter-tubule spaces where...
haematopoietic cells reside and adjacent to melanomacrophages (Fig. 6B). It must be noted that the polyclonal antibody used cross-reacted with both BATF3a and BATF3b as confirmed by Western blotting (Fig. 6).

3.5. Modulation of BATF3a and BATF3b expression in vitro

RTS-11 is a monocyte/macrophage like cell line derived from spleen. When stimulated with TLR ligands, PHA and type I and II rIFNs for 24 h, BATF3a was found to be induced by LPS, R848 and type II rIFNs but not by rFlagellin, polyI:C, PHA and type I rIFN (Fig. 7A and 7B). A moderate increase of BATF3b expression was also detected in cells treated with type II rIFN. The effects of TLR ligands were also examined at 6 h after stimulation and interestingly BATF3b was induced by rFlagellin, polyI:C and R848, with although weaker induction was seen for BATF3a with by rFlagellin and R848 (Fig. 7E). These results demonstrate that BATF3a and BATF3b are differently regulated differently by TLR ligands.—

Primary head kidney monocytes/macrophages were next used to investigate the modulation of BATF3s expression by TLR ligands and recombinant rIFNs (Fig. 7C, D). BATF3a expression was again up-regulated by LPS, R848 and rIFNγ, the latter to a very high fold increase. However, it was also induced by the other stimulants unlike the response in RTS-11 cells. In contrast, BATF3b was induced only by flagellin, polyI:C and rIFNγ.

In our previous study, recombinant trout IL-4/13A cultured cells derived from head kidney monocytes/macrophages expressed a high level of MHCII and a moderate level of putative DC markers such as CLEC4T1/DC-SIGN and CD83, and displayed a similar morphology to mammalian DCs, with dendrites on the cell surface (Wang, Wang et al., 2016; Johansson, Wang et al., 2016; Wang et al., 2018). Archived rIL-4/13A cultured primary head kidney monocyte/macrophage cDNA samples from TLR ligand treated cells were used to investigate the expression of BATF3 (Fig. 8). Interestingly, BATF3a and BATF3b were both significantly up-regulated in rIL-4/13A cultured cells by polyI:C and R848 (viral PAMPs) stimulation. BATF3a was also...
up-regulated to some extent by LPS treatment. No significant changes of BATF3b were detected in cells treated with LPS and Flagellin (bacterial PAMPs).

3.6. Modulation of BATF3a and BATF3b expression during infection

Enteric redmouth disease (ERM) is a serious septicaemic bacterial disease of salmonid fish species caused by infection with *Yersinia ruckeri* (Harun et al., 2011). A pathogenic strain (MT3072) of *Y. ruckeri* was used to infect trout by intraperitoneal injection (Wang et al., 2018). Expression of BATF3a and BATF3b was examined in systemic (spleen) and mucosal tissues (gills and intestine) tissues. A marked increase of transcripts was seen for both genes in spleen whilst they were moderately up-regulated in intestine (Fig. 9). No significant modulation of expression was detected for either gene in gills.

The expression of BATF3 genes was lastly examined in trout juveniles during infection with IHNV. IHNV is a member of the Rhabdoviridae family and infects salmonid juveniles. A recently identified IHNV stain (HLJ-09) was used to infect trout juveniles in this study. In head kidney, both BATF3a and BATF3b were up-regulated at days 3 and 5, with increases of >50-fold in the case of BATF3a at day 5 (Fig. 10). Expression of IHNV N and the antiviral gene Mx1 also increased in head kidney at days 3 and 5 post-injection (Fig. 10), verifying that infection had occurred.

4. Discussion

The BATF3 genes have not been described in non-mammalian vertebrates. In the present study, two BATF3 homologues (termed BATF3a and BATF3b) have been identified and characterized in rainbow trout (*Oncorhynchus mykiss*). BATF3a was reported previously, as constitutively expressed in CD8a+MHC II+ DC-like cells (Granja et al., 2015). We now show it is found to be more highly expressed in tissues such as spleen, liver, pyloric caeca and head kidney, and that both paralogues can be up-regulated (at least to some extent) in cultured cells by PAMPs and
cytokines, and in vivo by infection. Their potential role in fish immune responses is discussed below.

The BATF family consists of 3 members (BATF1-3) which are structurally related (Murphy et al., 2013). They contain a canonical α-helical bZIP domain and belong to the AP1 family which includes FOS, JUN and ATF. Unlike other vertebrates which have a single copy of the BATF3 gene, trout and salmon contain two BATF3 paralogues, with the predicted proteins sharing 78.3% sequence similarity. As seen in other vertebrates, both trout BATF3a and BATF3b reside next to the ATF gene in the genome (Fig. 1). Although trout BATF3b has a slightly higher sequence homology with tetrapod BATF3 than BATF3a, all the teleost fish homologues grouped in a single clade within which the salmonid BATF3 group split into the BATF3a and BATF3b subgroups. These data support the contention that duplication of BATF3 is a salmonid-specific event likely associated with the salmonid-specific whole genome duplication (Berthelot-Brunet et al., 2014).

The expression of BATF3 is limited to leucocytes in humans and mice, and increases during cDC development. The spleen is a major secondary lymphoid organ in fish, where leucocytes (e.g. antigen presenting cells, T and B cells) interact with each other. Therefore, it is not surprising that a high level of trout BATF3a transcripts was detected in this tissue (Fig. 5). Trout BATF3a was also highly expressed in pyloric caeca. Recent studies have shown that pyloric caeca is an important organ involved in mucosal immunity and harbors a high density of B and T cells amongst others (Ballesteros et al., 2013). That the BATF3 paralogues are constitutively expressed in immune tissues suggests they may be involved in maintenance of homeostasis of the immune system in fish.

Expression modulation of trout BATF3a and BATF3b was examined in primary monocytes/macrophages isolated from head kidney and a spleen monocyte/macrophage cell line (RTS-11). In the HK monocytes/macrophages, BATF3a could be induced by all the stimuli used. Notably, stimulation with IFNγ resulted in the largest increase of BATF3a expression (55-fold increase). In contrast to the HK monocytes/macrophages, only small changes in BATF3a and BAT3b
expression were seen largely unaltered in the RTS-11 cells, where relatively small increases were seen after stimulation with LPS, Flagellin, poly IC, R848 and rIFNγ, with modulation of BATF3b mainly seen at the earlier time (6 h) post-stimulaiton. It should be noted that the zinc finger transcription factors ZBTB46 and DC-SCRIPT/ZNF366 known to be involved in activation of antigen presenting cells, were down-regulated in primary HK monocytes/macrophages by rIFNγ, highlighting the central roles of rIFNγ in mediating antigen presentation in fish (Zou et al., 2005; Wang et al., 2018; Wiegertjes et al., 2016; Zou et al., 2005).

IL-4 in combination with granulocyte macrophage colony stimulating factor (GM-CSF) is commonly used for in vitro culture of dendritic cells in humans and mice (Dauer et al., 2003). Two IL-4/13 homologues (IL-4/13A and IL-4/13B) have been identified in rainbow trout but GM-CSF is absent in teleost fish (Zou and Secombes, 2011; Wang et al., 2016; Zou and Secombes, 2011). Trout rIL-4/13A can enhance HK monocyte/macrophage differentiation into CLEC4T+ cells (Johansson et al., 2016). These cells express a remarkably high level of MHC II molecules and moderate levels of the macrophage colony stimulating factor receptor (MCSFR) and CD83, and display a distinct morphology when stimulated with bacterial and viral PAMPs in association with inducible expression of ZBTB46 (Wang et al., 2018). In such cells, cultured with rIL-4/13A for 7 days, the transcript levels of BATF3a and BATF3b remained largely unchanged (7-10 x 10⁻⁴ of BATF3a relative to EF-1α, 1-3 x 10⁻⁴ of BATF3b relative to EF-1α) (Figs. 5 and 8), but further stimulation, in particular with viral PAMPs, enhanced expression several fold. The in vivo studies demonstrated that the BATF3 paralogues are also modulated during infection, in this case by bacterial and viral diseases. Relatively high increases in transcript level were seen in the target organs; spleen in the case of Yersiniosis and kidney in the case of IHNV. The kinetics of induction were also studied during the case of IHNV, where maximal increases were seen several days post-infection. Taken together, the present findings suggest that BATF3 genes may have both homeostatic and inducible functions within the immune system of fish, potentially in the context of DC differentiation and activation. The characterization of BATF genes
in fish provides useful data for further characterization of the role(s) of BATF3 in regulating leucocyte differentiation in early vertebrates.

Acknowledgements

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Figure legend

Fig. 1. Comparative analysis of gene synteny of BATF3. The rainbow trout WGS scaffolds were obtained from NCBI, and the gene synteny of other vertebrates was obtained from the Ensemble Genome Browser (http://sep2015.archive.ensembl.org/index.html). The arrows indicate the transcriptional direction. The homologous genes are shown with the same colour.

Fig. 2. Amino acid sequence alignment (A), and identity/similarity analysis (B) of BATF3 in Homo sapiens, Mus musculus, Gallus gallus, Chrysemys picta bellii, Xenopus tropicalis, Danio rerio, Oreochromis niloticus, Salmo salar and Oncorhynchus mykiss. Sequences were aligned using the ClustalW server (http://clustalw.ddbj.nig.ac.jp/). Sequence identity/similarity was analysed using the Pair-wise sequence alignment. Identity (*), strong similarity (:) and weak similarity (.) are indicated below the alignment. The structural domains of BATF3 were predicted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The core domain is boxed, with DNA binding domain (DB), hinge (H) and leucine zipper (LZ) regions indicated above the alignment. The conserved leucines are highlighted.

Fig. 3. Comparative analysis of BATF3 protein structure (A), tertiary structure (B) and identity/similarity of structural domains (C) between human (hu) and rainbow trout (rt). A, the structural domains of BATF3 molecules were predicted using the
Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The bZIP domain was constituted of a DNA binding domain, hinge region and leucine zipper region. B, the tertiary structure of bZIP domains was predicted using CPHmodels 3.2 Server (http://www.cbs.dtu.dk/services/CPHmodels/). C, domain identity/ similarity was analysed using the Pair-wise sequence alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Fig. 4. Phylogenetic tree analysis of BATF1-3. The phylogenetic tree was drawn using the Mega 6.0 software. The nNeighbour-joining algorithm was used as the clustering method and the distances matrix computed using the Poisson correction method. The tree was supported by 10,000 bootstrap repetitions using the Poisson model for amino acid substitution. The bootstrap values are indicated as percentages.

Fig. 5. Tissue distribution of rainbow trout BATF3. The expression levels of BATF3a and BATF3b were determined by real-time PCR and normalized to the expression level of a reference gene EF-1α. The results represent the means ± SE of five fish.

Fig. 6. Western blotting (A) and Immunohistochemical staining (B). Twenty µL of an IPTG-induced overnight culture of E. coli BL21 (DE3) cells transformed with pHISTEV-BATF3a (Lane 1) or pHISTEV-BATF3b (Lane 2) was analysed by Western blotting using a polyclonal antibody against the conserved leucine zipper region of human BATF3 (1:100, v/v) followed by a goat anti-rabbit IgG-peroxidase antibody (1:10,000 v/v) or the mouse monoclonal anti-polyhistidine-peroxidase antibody (Sigma, 1:2000, v/v). For immunohistochemistry, the kidney tissue (100-150 mg) of healthy trout was fixed using 4% paraformaldehyde (PFA) in sterile PBS and embedded into paraffin wax using standard histological methods. The slides were incubated with (right A) or without (left B) the rabbit polyclonal antibody against the conserved leucine zipper region of human BATF3 (1:100, v/v), and the cell nuclei were counterstained with Harris haematoxylin. Arrows indicate the positively-stained cells. Bar=50 µM.

Fig. 7. Modulation of expression of BATF3 in trout RTS-11 cells (A, B, E) and primary HK monocytes/macrophages (C, D). The cells were stimulated for 6 h (E) or
24 h (A, D–B) with LPS (20 μg/mL), rFlagellin (20 ng/mL), polyI:C (50 μg/mL), R848 (10 μg/mL), PHA (10 μg/mL), rIFN2 (type I) (20 ng/mL), rIFNγ (20 ng/mL) or PBS (control). The gene expression levels were determined by real-time PCR and normalized to that of EF-1α. The fold changes were calculated by comparing the average expression level of each treatment group with that of the respective control group. Bars indicate means ± SE of gene expression levels of cells from three flasks for RTS-11 cells (n=3) or four fish for the primary HK monocytes/macrophages (n=4).

* = p ≤ 0.05, ** = p ≤ 0.01.

Fig. 8. Expression modulation of BATF3 in trout HK monocytes/macrophages cultured with rIL-4/13A. The primary adherent HK leucocytes were isolated and cultured in the presence of 200 ng/mL rIL-4/13A for 7 days. The cells were then stimulated for 24 h with LPS (20 μg/mL), rFlagellin (20 ng/mL), polyI:C (50 μg/mL), R848 (10 μg/mL), or PBS (control). The gene expression levels were determined by real-time PCR and normalized to that of EF-1α. The fold changes were calculated by comparing the average expression level of each treatment group with that of the respective control group. Bars indicate means ± SE of gene expression levels of cells from four flasks. A, expression of BATF3a and BATF3b in HK monocytes/macrophages cultured with rIL-4/13A for 7 days; B, fold changes of expression of BATF3a and BATF3b after stimulation.

Fig. 9. Expression of BATF3, Mx1 and IHNV N in rainbow trout after infection with IHNV. The fish were challenged by intraperitoneal injection with a pathogenic strain (MT3072) of Y. ruckeri (5 × 10⁵ cfu per fish) or PBS. Gill, spleen and intestine were collected at 24 h post-challenge and real-time PCR was performed to determine BATF3 expression. The expression levels of BATF3a and BATF3b were normalized to that of EF-1α and fold changes calculated by comparing the expression level of challenged fish with that of the respective control fish (defined as 1). Bars indicate means ± SE of tissues from three fish. * = p ≤ 0.05.
kidney was collected for analysis of gene expression. Bars represent the fold change of expression level compared to that of uninfected control fish. Data shown are the means ± SE of 10 fish. * = p < 0.01 compared to day 1, # = p < 0.01 compared to day 3.

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Fig. 1

**Human (Homo sapiens)**
Chromosome 1: 212432887-212847649

**Mouse (Mus musculus)**
Chromosome 1: 191036639-191318194

**Chicken (Gallus gallus)**
Chromosome 3: 21114069-21291025

**Frog (Xenopus tropicalis)**
NW_004668237.1: 112383290-112841079

**Zebrafish (Danio rerio)**
Chromosome 20: 37857614-37924504

**Cave fish (Astyanax mexicanus)**
Chromosome KB882998.1: 3896478-4004315

**Amazon molly (Poecilia formosa)**
Chromosome K1519970.1: 471807-538412

**Rainbow trout (Oncorhynchus mykiss)**
Scaffold_324 (CCAfO10027628): 802893-880777
Scaffold_1368 (CCAfO10060656): 138804-175945

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**Mammalia**

**Aves**

**Amphibia**

**Teleostei**
Fig. 3

A

N-terminal 0 20 40 60 80 100 120 127
C-terminal Amino acid (AA)

huBATF3
rtBATF3a
rtBATF3b

DNA binding domain
hinge
leucine zipper

B

Pro 32  Asp 29  Asp 32
Glu 97  Ala 94  Ala 97

huBATF3  rtBATF3a  rtBATF3b

C

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<tr>
<th>N-terminal</th>
<th>bZIP</th>
<th>C-terminal</th>
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<td>32 (1-32)</td>
<td>65 (33-97)</td>
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<tr>
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<td>29 (1-29)</td>
<td>65 (30-94)</td>
</tr>
<tr>
<td>rtBATF3b (AA)</td>
<td>32 (1-32)</td>
<td>65 (33-97)</td>
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Pair-wise identity/similarity

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<tr>
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<tbody>
<tr>
<td>rtBATF3a &amp; huBATF3</td>
<td>5.9% / 9.8%</td>
<td>55.4% / 78.5%</td>
<td>36.4% / 42.4%</td>
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<td>3.4% / 3.4%</td>
<td>61.5% / 83.1%</td>
<td>37.5% / 50.0%</td>
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<tr>
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<td>75.4% / 86.2%</td>
<td>71.9% / 71.9%</td>
</tr>
</tbody>
</table>
Fig. 4
Fig. 5

Expression relative to EF-1α (× 10^-4)

- BATF3a
- BATF3b
Fig. 6

A

1      2

α-BATF3

B
Fig. 7

A  

B  

C  

D
Fig. 8

A

B
Fig. 9

Bar chart showing fold change for BATF3a and BATF3b in gills, spleen, and intestine. The chart indicates statistically significant differences (*) between BATF3a and BATF3b in the spleen and intestine.
Fig. 10

head kidney

Fold change relative to control group

- BATF3a
- BATF3b
- Mx1
- IHNV N

1 day
3 day
5 day
Supplementary material for online publication only
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