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

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

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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 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.

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

***Rebuttal Note**

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 IFNgR1 / 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!

-How the authors have verified that EF1a was an adequate house-keeping gene for these studies should be mentioned.

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 conclusoon should be play 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.

*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.

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

2

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

26

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

31 zipper; PAMP, pathogen-associated molecular pattern; PHA, phytohaemagglutinin;
32 polyI:C, polyinosinic:polycytidylic acid; TLR, Toll-like receptor.
33

34 **ABSTRACT**

35 Basic leucine zipper transcription factor ATF-like (BATF) -3 is a member of the
36 activator protein 1 (AP-1) family of transcription factors and is known to play a vital
37 role in regulating differentiation of antigen-presenting cells in mammals. In this study,
38 two BATF3 homologues (termed BATF3a and BATF3b) have been identified in
39 rainbow trout (*Oncorhynchus mykiss*). Both genes were constitutively expressed in
40 tissues, with particularly high levels of BATF3a in spleen, liver, pyloric caecae and
41 head kidney. BATF3a was also more highly induced by PAMPs and cytokines in
42 cultured cells, with type II IFN a particularly potent inducer. In rIL-4/13
43 pre-stimulated cells, the viral PAMPS polyI:C and R848 had the most pronounced
44 effect on BATF3 expression. BATF3 expression could also be modulated in vivo,
45 following infection with *Yersinia ruckeri*, a bacterial pathogen causing redmouth
46 disease in salmonids, or with the rhabdovirus IHNV. The results suggest that BATF3
47 may be functionally conserved in regulating the differentiation and activation of
48 immune cells in lower vertebrates and could be explored as a potential marker for
49 comparative investigation of leucocyte lineage commitment across the vertebrate
50 phyla.
51

52 **Highlights**

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53 1. The BATF3 genes were analysed in detail for the first time in fish.
54 2. Trout BATF3a is highly expressed in spleen, liver and pyloric caeca.
55 3. Trout BATF3a is highly up-regulated in monocytes/macrophages by IFNγ
56 treatment.
57 4. Both BATF3a and BATF3b are induced after infection with bacterial and viral
58 diseases.
59 ~~1. The BATF3 genes were identified for the first time in fish.~~
60 ~~2. 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~~of bacterial and 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 kn~~sh~~own 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~~, and~~ encode~~s~~ a protein of 127 aa and 118 aa respectively, that share high homology (80% similarity),~~. It~~ lack~~s~~ a signal peptide and bind~~s~~ 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 t~~The BATF3 gene is expressed ~~only~~ mainly in immune cells ~~originating in~~of hematopoietic organs (Williams et al., 2001), and in particular~~. Expression of BATF3 is mainly studied~~ in dendritic cells (DCs). For example, i~~I~~t can be~~is at~~ detected~~able levels~~ in ~~the~~ common ~~dendritic cell (~~DC~~)~~ 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

91 and Th17 cells also express BATF3 (Hildner et al., 2008).
92 The central roles of BATF3 in orchestrating leucocyte lineage commitment have
93 drawn significant attention in recent years. Emerging evidence indicates that BATF3
94 together with other members of the BATF family play critical roles in regulating
95 leucocyte differentiation, especially in directing the commitment of DC precursors
96 into specific lineages. Gene-knockout studies in mice demonstrate that BATF3 is
97 indispensable for the development of cDCs. The Batf3−/− mice do not develop
98 CD8α+ cDCs which are required for cytotoxic T cell immunity and antiviral defence
99 (Hildner et al., 2008; Sun et al., 2017). In adult mice, the intestinal BATF3-dependent
100 cDCs are required for homeostasis and antiviral T-cell immunity (Edelson~~, KC~~ et al.,
101 2010; Sun et al., 2017). Further, tissue-resident BATF3-dependent CD103+ DCs once
102 activated can produce ~~a~~ large amounts of interleukin (IL)-12, promoting a local Th1
103 response to combat *Leishmania major* infection (Martinez-Lopez et al., 2015).
104 However, other members of the BATF3 family may also be involved in regulation of
105 immune responses. Recent studies indicate that the roles of BATF3 in promoting
106 expansion of functional CD8+ cDCs to control infection of intracellular pathogens
107 may be compensated by other members of the BATF family via the interaction of the
108 conserved LZ domain with IRF4 or IRF8 (Tussiwand et al., 2012).

109 A recent study has shown that in rainbow trout (*Oncorhynchus mykiss*) skin
110 CD8a+MHC II+ DC-like cells constitutively express BATF3. This finding is
111 interesting and implies that ~~the~~ BATF3 may have conserved roles during vertebrate
112 evolution (Granja et al., 2015). Since no further analysis of BATF3 has been
113 undertaken to date, ~~I~~in this study we determined initially whether other BATF3
114 paralogues exist in teleost/salmonid fish, as a consequence of the 3rd or 4th whole
115 genome duplication events seen in these species, and analysed the phylogeny of
116 BATF3 in the context of vertebrate phyla. T~~, t~~wo BATF3 homologues were identified
117 in rainbow trout ~~(*Oncorhynchus mykiss*)~~ and their ~~the phylogeny of BATF3 was~~
118 ~~studied in the context of vertebrate phyla.~~ ~~E~~expression ~~of the trout BATF3~~
119 ~~paralogues~~ was studied in vivo after bacterial and viral infection and in vitro in
120 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~~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~~ 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~~e~~-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 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, *Eco*RI (New England Biolabs, UK). Plasmids were sequenced by Eurofins MWG Operon.

2.3. Bioinformatics analyses

The CDS regions and deduced amino acid sequences of BATF3 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~~for~~ examine~~ing~~ 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~~ed in~~ during RNA

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 °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~~A~~ previously ~~study showed~~ that EF-1α~~a~~ i~~wa~~s 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 cm^2 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.

244

245 *2.6. Expression of BATF3 in primary head kidney monocytes/macrophages and*
246 *rIL-4/13A cultured monocytes/macrophages cells treated withby TLR ligands and/or*
247 *interferons*

248

249 The primary head kidney (HK) monocytes/macrophages were isolated from freshly
250 killed rainbow trout using the method described previously by Peddie et al. (Peddie et
251 al., 2001). Briefly, fish were anaesthetised, killed, and the anterior kidney removed
252 aseptically and passed through a 100 µm nylon mesh using L-15 medium
253 supplemented with P/S, heparin (10 units/mL), and 2% FCS. After centrifugation at
254 400 x g for 10 min at 4°C, the primary HK cells were resuspended in incomplete cell
255 culture medium (L-15, P/S, 0.1% FCS) and washed once. The cell suspension (5×10^6
256 cells) was seeded into 25 cm^2 flasks containing incomplete cell culture medium (L-15,
257 P/S, 0.1% FCS) and incubated at 20-°C overnight. The unattached cells were carefully
258 removed and complete medium (L-15, P/S, 10% FCS) was added to the flasks. The
259 adherent cells (mostly monocytes/macrophages) were stimulated with LPS, rFlagellin,
260 polyI:C, R848, PHA, rIFN2, rIFNγ or PBS for 24 h as described above and then
261 harvested for real-time PCR analysis.

262 Archived cDNA samples from rIL-4/13A-cultured primary HK
263 monocytes/macrophages were analysed for the expression level of BATF3 (Wang et
264 al., 20186). Briefly, the adherent primary HK monocytes/macrophages cells were
265 cultured in complete medium containing 200 ng/mL of rIL-4/13A. At days 1, 3 and 5,
266 the medium was replaced with fresh complete medium containing 200 ng/mL
267 rIL-4/13A. At day 7, the cells were stimulated with LPS, rFlagellin, polyI:C or R848.
268 After 24 h, the cells were harvested for gene expression analysis by real-time PCR.

269

270 *2.7. Expression of BATF3 in spleen, gills and intestine during Y. ruckeri infection*

271

272 The expression of BATF3 was determined in archived cDNA samples of spleen,
273 gills and intestine taken from rainbow trout after intraperitoneal injection with a

274 pathogenic strain (MT3072) of *Y. ruckeri* (0.5 mL/fish, 1 × 10^6 cfu/mL) or 0.5 mL of
275 PBS as control (Gorgoglione Wang et al., 2018et al., 2016 – BUT THIS PAPER IS
276 BROWN TROUT!). Tissues from three fish were taken at 24 h post-challenge and
277 analyzed by real-time PCR.
278

279 *2.8. Expression of BATF3 in kidney during IHNV infection*

280

281 Six groups of 10 healthy rainbow trout (weighing ~3 g, each group containing 10
282 fish) were used for the IHNV challenge experiment. Preparation of IHNV (strain
283 HLJ-09) was described previously (Wang et al., 2016a; Wang et al., 2016b???).
284 Thirty fFish were injected intraperitoneally with 50 μL of L-15 medium containing 1
285 x 10^5 pfu of IHNV. Mock-infected control groups were injected with PBS only. Head
286 kidney was collected from infected and control fish at days 1, 3 and 5 (10 fish per
287 group) for extraction of total RNA, using an Omega Bio-Tek extraction kit I (Omega
288 Bio-Tek, Doraville, GA, USA) following the manufacturer's instructions. cDNA was
289 synthesised using oligo(dT)15 (Takara, Japan) and a Superscript Reverse
290 Transcriptase Reagent Kit (Takara, Japan). Real-time PCR was performed using
291 SYBR Premix EX Taq Ⅱ (Takara, Japan) on the ABI 7500 real-time PCR system
292 (Applied biosystems, Carlsbad, CA, USA) using the following conditions: 1 cycle of
293 30 s at 95 °C, 40 cycle of 3 s at 95 °C, and 30 s at 60 °C. The average cycle threshold
294 (Ct) was calculated from triplicate measurements using the instrument's software in
295 "auto Ct" mode (ABI 7500 system, version 2.3). Relative Ct values of three
296 independent tests were calculated by the $2^{-\Delta\Delta Ct}$ method. EF-1α was used as an internal
297 reference for normalization of gene expression. Infection of VHSV IHNV was
298 verified by examining expression of the IHNV N gene and Mx gene by real-time PCR.
299 The primers for the IHNV N gene and Mx gene awere listed in supplementary Table
300 1.

301

302 *2.9. Immunohistochemical staining*

303 AThe rabbit polyclonal antibody against the conserved leucine-zipper region of

304 human BATF3 (Merck, Cat. No. ABE1007) was used in i~~I~~mmunohistochemical
305 staining. To verify the cross-reactivity of this~~human polyclonal BATF3~~ antibody with
306 trout BATF3a and BATF3b, the full length cDNA fragments were amplified and
307 cloned into the pHISTEV vector (kindly provided by Dr Hai Deng, University of
308 Aberdeen) at the *Bam*HI/*Hin*dIII sites. The resultant plasmids (pHISTEV-BATF3a and
309 pHISTEV-BATF3b) were transformed into *E. coli* BL21 (DE3) ~~cells~~. The cells were
310 then induced by 2 mM IPTG overnight at 37°C in a shaker (150 rpm) and 20 µL of
311 cell culture collected for SDS-PAGE gel electrophoresis and Western blotting. The
312 rabbit anti human BATF3 polyclonal antibody and the secondary goat anti-rabbit
313 IgG-peroxidase antibody (Sigma) were diluted ~~by~~ 1:100 (v/v) and 1:10,000 (v/v)
314 respectively. Since the recombinant proteins have a 6-histidine tag at the N- terminus,
315 a~~the~~ mouse monoclonal anti-polyhistidine-peroxidase antibody (Sigma, 1:2,000, v/v)
316 was used to validate the recombinant trout BATF3a and BATF3b detected by the
317 human BATF3 antibody.

318 Next~~The~~ kidney tissue (100-150 mg) from~~of~~ healthy trout was fixed using 4%
319 paraformaldehyde (PFA) in sterile PBS for 20 h at 4°C followed by 5 washes in sterile
320 PBS. Tissue was incubated in the final PBS wash for 1 h at 4°C and stored at 4°C in
321 70% ethanol prior to further tissue processing. Tissue was embedded into paraffin
322 wax using standard histological methods
323 (http://www.ihcworld.com/_protocols/histology/paraffin_section.htm).
324 Immunohistochemistry was performed using a Dako autostainer E 172566 (Model:
325 LV-1, Dako, UK) as described previously (Alnabulsi et al., 2017; Swan et al., 2016).
326 The tissue sections were first dewaxed in xylene for a minimum of 10 min and
327 rehydrated by immersion in decreasing ethanol concentrations. Then, antigen retrieval
328 was performed by heating the tissue sections for 20 min in a microwave (800 W)
329 while sections were fully immersed in 10 mM citrate buffer (pH 6.0). After cooling,
330 the sections were incubated with or without (negative control) a rabbit polyclonal
331 antibody against the conserved leucine-zipper region of human BATF3 (1:100, v/v~~,~~
332 ~~Merck, Cat. No. AB1007~~) for 60 min at room temperature. The sections were then
333 washed twice with washing buffer (Dako), ~~blocked~~ incubated with blocking solution

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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. A~~n a~~ntibody 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

al., 2001). 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 ~~It must be noted that the~~ polyclonal antibody against the conserved leucine-zipper region of human BATF3 was used, and that it~~used~~ 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

424 h~~a~~ematopoietic cells reside and adjacent to melano-macrophages (Fig. 6B). ~~It must be~~
425 ~~noted that the polyclonal antibody used cross-reacted with both BATF3a and BATF3b~~
426 ~~as confirmed by Western blotting (Fig. 6)~~
427
428 *3.5. Modulation of BATF3a and BATF3b expression in vitro*
429
430 RTS-11 is a monocyte/macrophage like cell line derived from spleen. When
431 stimulated with TLR ligands, PHA and type I and II rIFNs for 24 h, BATF3a was
432 found to be induced by LPS, R848 and type II rIFN~~s~~ but not by rF~~f~~lagellin, polyI:C,
433 PHA and type I rIFN (Fig. 7A and 7B). A moderate increase of BATF3b expression
434 was also detected in cells treated with type II rIFN. The effects of TLR ligands were
435 also examined at 6 h after stimulation and interestingly BATF3b was induced by
436 rF~~f~~lagellin, polyI:C and R848, with ~~although~~ weaker induction ~~was~~ seen for BATF3a
437 with~~by~~ rF~~f~~lagellin and R848 (Fig. 7E). These results demonstrate that BATF3a and
438 BATF3b are ~~differently~~ regulated differently by TLR ligands.—
439 Primary head kidney monocytes/macrophages were next used to investigate the
440 modulation of BATF3s expression by TLR ligands and ~~recombinant~~ rIFNs (Fig. 7C,
441 D). BATF3a expression was again up-regulated by LPS, R848 and rIFNγ, the latter to
442 a very high fold increase. However, it was also induced by the other stimulants unlike
443 the response in RTS-11 cells. In contrast, BATF3b was induced only by flagellin,
444 polyI:C and rIFNγ.
445 In our previous study, recombinant trout IL-4/13A cultured cells derived from head
446 kidney monocytes/macrophages expressed a high level of MHCII and a moderate
447 level of putative DC markers such as CLEC4T1/DC-SIGN and CD83, and displayed a
448 similar morphology to mammalian DCs, with dendrites on the cell surface (~~Wang,~~
449 ~~Wang et al., 2016~~;~~,~~ Johansson~~, Wang~~ et al., 2016; Wang et al., 2018). Archived
450 rIL-4/13A cultured primary head kidney monocyte/macrophage cDNA samples from
451 TLR ligand treated cells were used to investigate the expression of BATF3 (Fig. 8).
452 Interestingly, BATF3a and BATF3b were both significantly up-regulated in rIL-4/13A
453 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 F~~f~~lagellin (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~~but~~ both paralogues can be~~were~~ 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 rIFNγ 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

514 expression were seen~~was largely unaltered~~ in the RTS-11 cells, ~~where relatively small~~
515 ~~increases were seen~~ after stimulation with LPS, Flagellin, poly I:C, R848 and rIFNγ,
516 with modulation of BATF3b mainly seen at the earlier time (6 h) post-stimulaiton. It
517 should be noted that the zinc finger transcription factors ZBTB46 and
518 DC-SCRIPT/ZNF366 known to be involved in activation of antigen presenting cells,
519 were down-regulated in primary HK monocytes/macrophages by rIFNγ, highlighting
520 the central roles of rIFNγ in mediating antigen presentation in fish (~~Zou et al., 2005;~~
521 Wang et al., 2018~~6c~~; Wiegertjes et al., 2016; Zou et al., 2005).

522 IL-4 in combination with granulocyte macrophage colony stimulating factor
523 (GM-CSF) is commonly used for in vitro culture of dendritic cells in humans and
524 mice (Dauer et al., 2003). Two IL-4/13 homologues (IL-4/13A and IL-4/13B) have
525 been identified in rainbow trout but GM-CSF is absent in teleost fish (~~Zou and~~
526 ~~Secombes, 2011;~~ Wang et al., 2016c; Zou and Secombes, 2011~~d~~). Trout rIL-4/13A can
527 enhance HK monocyte/macrophage differentiation into CLEC4T+ cells (Johansson et
528 al., 2016). These cells express a remarkably high level of MHC II molecules and
529 moderate levels of the macrophage colony stimulating factor receptor (MCSFR) and
530 CD83, and display a distinct morphology when stimulated with bacterial and viral
531 PAMPs in association with inducible expression of ZBTB46 (Wang et al., 2018~~6c~~). In
532 such cells, cultured with rIL-4/13A for 7 days, the transcript levels of BATF3a and
533 BATF3b remained largely unchanged (7-10 x 10^{-4} of BATF3a relative to EF-1α, 1-3 x
534 10^{-4} of BATF3b relative to EF-1α) (Figs. 5 and 8), but further stimulation, in
535 particular with viral PAMPs, enhanced expression several fold.

536 The in vivo studies demonstrated that the BATF3 paralogues are also modulated
537 during infection, in this case by bacterial and viral diseases. Relatively high increases
538 in transcript level were seen in the target organs; spleen in the case of Yersiniosis and
539 kidney in the case of IHNV. The kinetics of induction ~~were~~ was also studied during~~in~~
540 ~~the~~ case of IHN~~V~~, where maximal increases were seen several days post-infection.
541 Taken together, the present findings suggest that BATF3 genes may have both
542 homeostatic and inducible functions within the immune system of fish, potentially in
543 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

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 n~~N~~eighbour-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 ~~I~~immunohistochemical staining (B). Twenty uL of an IPTG-induced overnight culture of *E. coli* BL21 (DE3) cells transformed with pHISTEV-BATF3a (Lane 1) or pHISTEV-BATF3b~~a~~ (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). * = $p \leq 0.05$, ** = $p \leq 0.01$.

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. * = $p \leq 0.05$, ** = $p \leq 0.01$. 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. Gills, spleen and intestine were collected at 24 h post-challenge and real-time PCR was~~ere~~ 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. * = $p \leq 0.05$.

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 1×10^5 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

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 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.

Highlights

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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.~~

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

4. Both BATF3a and BATF3b are induced after infection withof bacterial and 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 knshown 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, and encodes 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 tThe BATF3 gene is expressed only mainly in immune cells originating inof hematopoietic organs (Williams et al., 2001), and in particular. Expression of BATF3 is mainly studied in dendritic cells (DCs). For example, iIt can beis at detectedable levels in the common dendritic cell (DC) precursors such as monocytes (which also maturedevelop into macrophages) and isbut 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

91 and Th17 cells also express BATF3 (Hildner et al., 2008).

92 The central roles of BATF3 in orchestrating leucocyte lineage commitment have
93 drawn significant attention in recent years. Emerging evidence indicates that BATF3
94 together with other members of the BATF family play critical roles in regulating
95 leucocyte differentiation, especially in directing the commitment of DC precursors
96 into specific lineages. Gene-knockout studies in mice demonstrate that BATF3 is
97 indispensable for the development of cDCs. The Batf3−/− mice do not develop
98 CD8α+ cDCs which are required for cytotoxic T cell immunity and antiviral defence
99 (Hildner et al., 2008; Sun et al., 2017). In adult mice, the intestinal BATF3-dependent
100 cDCs are required for homeostasis and antiviral T-cell immunity (Edelson~~, KC~~ et al.,
101 2010; Sun et al., 2017). Further, tissue-resident BATF3-dependent CD103+ DCs once
102 activated can produce ~~a~~ large amounts of interleukin (IL)-12, promoting a local Th1
103 response to combat *Leishmania major* infection (Martinez-Lopez et al., 2015).
104 However, other members of the BATF3 family may also be involved in regulation of
105 immune responses. Recent studies indicate that the roles of BATF3 in promoting
106 expansion of functional CD8+ cDCs to control infection of intracellular pathogens
107 may be compensated by other members of the BATF family via the interaction of the
108 conserved LZ domain with IRF4 or IRF8 (Tussiwand et al., 2012).

109 A recent study has shown that in rainbow trout (*Oncorhynchus mykiss*) skin
110 CD8a+MHC II+ DC-like cells constitutively express BATF3. This finding is
111 interesting and implies that ~~the~~ BATF3 may have conserved roles during vertebrate
112 evolution (Granja et al., 2015). Since no further analysis of BATF3 has been
113 undertaken to date, ~~I~~in this study we determined initially whether other BATF3
114 paralogues exist in teleost/salmonid fish, as a consequence of the 3rd or 4th whole
115 genome duplication events seen in these species, and analysed the phylogeny of
116 BATF3 in the context of vertebrate phyla. T~~, t~~wo BATF3 homologues were identified
117 in rainbow trout ~~(*Oncorhynchus mykiss*)~~ and their ~~the phylogeny of BATF3 was~~
118 ~~studied in the context of vertebrate phyla.~~ ~~E~~expression ~~of the trout BATF3~~
119 ~~paralogues~~ was studied in vivo after bacterial and viral infection and in vitro in
120 cultured monocytes/macrophages after stimulation with TLR ligands, a lectin and

121 interferons (IFNs). The results provide a first insight into the evolution of BATF3 in
122 lower vertebrates and will help develop potential comparative markers to study
123 leucocyte ~~lineage commitment~~differentiation between fish and higher vertebrates.
124

125 **2. Materials and methods**

126

127 *2.1. Fish*

128

129 Rainbow trout (*Oncorhynchus mykiss*) weighing~~(approximately~~ ~100 g~~)~~ were
130 maintained in 1 m diameter tanks supplied with a continuous flow of recirculating
131 freshwater at 15 ± 1°C in the aquarium facilities in the Zoology building, University
132 of Aberdeen. Fish were fed with commercial trout pellets (EWOS) and acclimated to
133 aquarium conditions for at least 2 weeks before use. Fish were anaesthetised using
134 2-phenoxyethanol (0.05%, Sigma Aldrich) and killed by subsequent destruction of the
135 brain prior to tissue harvest. All experiments at Aberdeen were carried out under the
136 UK Home Office project license PPL 60/4013. For the ~~IHNV (~~infectious
137 hematopoietic necrosis virus (IHNV) challenge experiment, rainbow trout weighing
138 ~3 g were obtained from the cold-water fish experiment station (Mudanjiang, China)
139 and maintained in 120 cm × 50 cm × 60 cm tanks with aeration at 16~~-~~°C. The fish
140 were fed daily with a dry pellet food and were also acclimated to aquarium~~laboratory~~
141 conditions for at least~~2~~ 2 weeks before use. The experiment was undertaken according
142 to the guidance of the local animal ethics committee.
143
144

145 *2.2. RNA extraction, cDNA synthesis and gene cloning*

146

147 The trout tissues and cells were collected for extraction of total RNA using TRI
148 Reagent® (Sigma~~e~~-Aldrich, UK) according to the manufacturer's instructions. cDNA
149 was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo
150 Scientific, UK). The cDNA samples were kept at -20°C before use.

151 The human BATF3 sequence (GenBank Acc. No., NP_061134) was used as the bait
152 sequence to undertake ~~the~~ BLAST (tBLASTn) analysis of the Whole-genome shotgun
153 (WGS) database, transcriptome shotgun assembly (TSA) database and expressed
154 sequence tags (ESTs) database, to obtain the trout BATF3 sequences. The WGS
155 contigs were retrieved and analyzed for prediction of coding sequences using the
156 GenScan program (Burge and Karlin, 1997). Predicted potential coding DNA
157 sequence (CDS) were confirmed for sequence similarity by the BLASTp analysis in
158 the non-redundant protein sequence database. Two WGS contigs (Accession Nos.,
159 CCAF010027628.1 and CCAF010060656.1) were identified to contain homologues
160 of BATF3 genes and contained complete coding sequences (CDS) and untranslated
161 regions (UTRs). Primers (supplementary Table 1) located in the 5' and 3' UTRs were
162 designed for amplification of full length cDNA using trout head kidney cDNA as
163 template. The PCR reaction volume was 25 μL including 2 μL of each of the primers
164 (10 μM), 2 μL of cDNA, 5 μL of 5 × MyFi Reaction Buffer, 13 μL of PCR water and
165 1 μL MyFi DNA Polymerase (Bioline, UK). The PCR reaction conditions were
166 performed using the following program: 95~~-~~°C for 3 min, followed by 35 cycles at 95~~-~~°C
167 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.
168 The purified PCR products were cloned into the pGEM®-T Easy cloning vector
169 (Promega, UK) and transformed into RapidTrans™ TAM1 competent *Escherichia*
170 *coli* cells (Active Motif, Belgium). The transformed cells were cultured on LB agar
171 plates (Sigma-Aldrich, UK) with ampicillin (100 μg/mL) overnight at 37~~-~~°C and
172 colonies were screened by colony PCR using the vector specific primer M13F and a
173 gene specific primer (supplementary Table 1~~.~~). Plasmid DNA was purified using a
174 QIAprep® spin DNA miniprep kit (QIAGEN, UK) according to the manufacturer's
175 instructions and the size of the inserts was verified by digestion with the restriction
176 enzyme, *Eco*RI (New England Biolabs, UK). Plasmids were sequenced by Eurofins
177 MWG Operon.

178

179 *2.3. Bioinformatics analyses*

180

181 The CDS regions and deduced amino acid sequences of BATF3~~s~~ were analyzed
182 using the ExPASy Translate tool (http://web.expasy.org/translate/) and the homology
183 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~~for~~ examine~~ing~~ 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~~ed in~~ during RNA

214 preparation. The PCR reaction consisted of 2 µL of primers (10 pmol each), 4 mL of
215 cDNA and 14 µL of PCR mix. The program was as follows: 10 min at 95~~-~~°C for
216 enzyme activation, 40 amplification cycles (95~~-~~°C for 30 s, 60-63~~-~~°C for 30 s and 72~~-~~°C
217 for 30 s), followed by 5 s at 90~~-~~°C to obtain the melting curve. The serially diluted
218 purified PCR products were used ~~as standards to serve~~ as reference to establish
219 standard curves for quantification in each 96-well plate. The relative expression level
220 of the BATF3 was calculated as arbitrary units and normalised against the expression
221 level of rainbow trout elongation factor (EF)-1α. It has been shown~~A~~ previously ~~study~~
222 ~~showed~~ that EF-1α~~a~~ i~~wa~~s one of the suitable house-keeping genes for use in salmonid
223 gene expression studies, as seen in studies of viral infection in Atlantic salmon
224 where~~whose~~ expression was not altered substantially ~~during viral infection~~ (Løvoll et
225 al., 2011).
226
227 *2.5. Expression of BATF3 in RTS-11 cells treated by TLR ligands and interferons*~~.~~
228
229 The expression of BATF3 was studied in the mononuclear/macrophage -like cell
230 line, RTS-11, from rainbow trout spleen (Brubacher~~, Secombes~~ et al., 2000). The cells
231 were maintained in Leibovitz (L-15) medium (Invitrogen, UK) containing 30% fetal
232 calf serum (FCS; Labtech International, UK) and antibiotics (100 U/mL penicillin and
233 100 mg/mL streptomycin; P/S; Invitrogen, UK) at 20~~-~~°C. Five mL of cells
234 (approximately 1×10^6 cells) were seeded into 25 cm^2 flasks (Sarstedt, Germany),
235 cultured overnight and then stimulated for 6 or 24 h with LPS (20 µg/mL; *E. coli*
236 strain 055:B5; Sigma-Aldrich, UK), recombinant *Yersinia ruckeri* Flagellin (rFlagellin)
237 (20 ng/mL) (Wangkahart et al., 2016), polyI:C (50 µg/mL; Sigma-Aldrich, UK), R848
238 (10 µg/mL; Sigma-Aldrich, UK), PHA (10 µg/mL; Sigma-Aldrich, UK), recombinant
239 interferon 2 (rIFN2) (20 ng/mL) (Zou et al., 2007), rIFNγ (20 ng/mL) (Zou~~,~~ et al.,
240 2005) or phosphate buffered saline (PBS) as control. The purity and activities of
241 recombinant cytokines were evaluated previously (Zou et al., 2005; 2007; Gao et al.,
242 2009; Skjesol et al., 2010; Wangkahart et al., 2016~~; Zou, et al., 2005; 2007~~). Real-time
243 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 withby 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 (5×10^6 cells) was seeded into 25 cm^2 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., 20186). 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

274 pathogenic strain (MT3072) of *Y. ruckeri* (0.5 mL/fish, 1 × 10^6 cfu/mL) or 0.5 mL of
275 PBS as control (Gorgoglione Wang et al., 2018et al., 2016 – BUT THIS PAPER IS
276 BROWN TROUT!). Tissues from three fish were taken at 24 h post-challenge and
277 analyzed by real-time PCR.
278
279 *2.8. Expression of BATF3 in kidney during IHNV infection*
280
281 Six groups of 10 healthy rainbow trout (weighing ~3 g, each group containing 10
282 fish) were used for the IHNV challenge experiment. Preparation of IHNV (strain
283 HLJ-09) was described previously (Wang et al., 2016a; Wang et al., 2016b???).
284 Thirty fFish were injected intraperitoneally with 50 µL of L-15 medium containing 1
285 x 10^5 pfu of IHNV. Mock-infected control groups were injected with PBS only. Head
286 kidney was collected from infected and control fish at days 1, 3 and 5 (10 fish per
287 group) for extraction of total RNA, using an Omega Bio-Tek extraction kit I (Omega
288 Bio-Tek, Doraville, GA, USA) following the manufacturer's instructions. cDNA was
289 synthesised using oligo(dT)15 (Takara, Japan) and a Superscript Reverse
290 Transcriptase Reagent Kit (Takara, Japan). Real-time PCR was performed using
291 SYBR Premix EX Taq Ⅱ (Takara, Japan) on the ABI 7500 real-time PCR system
292 (Applied biosystems, Carlsbad, CA, USA) using the following conditions: 1 cycle of
293 30 s at 95-°C, 40 cycle of 3 s at 95-°C, and 30 s at 60-°C. The average cycle threshold
294 (Ct) was calculated from triplicate measurements using the instrument's software in
295 "auto Ct" mode (ABI 7500 system, version 2.3). Relative Ct values of three
296 independent tests were calculated by the $2^{-\Delta\Delta Ct}$ method. EF-1α was used as an internal
297 reference for normalization of gene expression. Infection of VHSV IHNV was
298 verified by examining expression of the IHNV N gene and Mx gene by real-time PCR.
299 The primers for the IHNV N gene and Mx gene awere listed in supplementary Table
300 1.
301
302 *2.9. Immunohistochemical staining*
303 AThe rabbit polyclonal antibody against the conserved leucine-zipper region of

304 human BATF3 (Merck, Cat. No. ABE1007) was used in i~~I~~mmunohistochemical
305 staining. To verify the cross-reactivity of this~~human polyclonal BATF3~~ antibody with
306 trout BATF3a and BATF3b, the full length cDNA fragments were amplified and
307 cloned into the pHISTEV vector (kindly provided by Dr Hai Deng, University of
308 Aberdeen) at the *Bam*HI/*Hin*dIII sites. The resultant plasmids (pHISTEV-BATF3a and
309 pHISTEV-BATF3b) were transformed into *E. coli* BL21 (DE3)~~-cells~~. The cells were
310 then induced by 2 mM IPTG overnight at 37°C in a shaker (150 rpm) and 20 µL of
311 cell culture collected for SDS-PAGE gel electrophoresis and Western blotting. The
312 rabbit anti human BATF3 polyclonal antibody and the secondary goat anti-rabbit
313 IgG-peroxidase antibody (Sigma) were diluted ~~by~~ 1:100 (v/v) and 1:10,000 (v/v)
314 respectively. Since the recombinant proteins have a 6-histidine tag at the N- terminus,
315 a~~the~~ mouse monoclonal anti-polyhistidine-peroxidase antibody (Sigma, 1:2,000, v/v)
316 was used to validate the recombinant trout BATF3a and BATF3b detected by the
317 human BATF3 antibody.

318 Next~~The~~ kidney tissue (100-150 mg) from~~of~~ healthy trout was fixed using 4%
319 paraformaldehyde (PFA) in sterile PBS for 20 h at 4°C followed by 5 washes in sterile
320 PBS. Tissue was incubated in the final PBS wash for 1 h at 4°C and stored at 4°C in
321 70% ethanol prior to further tissue processing. Tissue was embedded into paraffin
322 wax using standard histological methods
323 (http://www.ihcworld.com/_protocols/histology/paraffin_section.htm).

324 Immunohistochemistry was performed using a Dako autostainer E 172566 (Model:
325 LV-1, Dako, UK) as described previously (Alnabulsi et al., 2017; Swan et al., 2016).
326 The tissue sections were first dewaxed in xylene for a minimum of 10 min and
327 rehydrated by immersion in decreasing ethanol concentrations. Then, antigen retrieval
328 was performed by heating the tissue sections for 20 min in a microwave (800 W)
329 while sections were fully immersed in 10 mM citrate buffer (pH 6.0). After cooling,
330 the sections were incubated with or without (negative control) a rabbit polyclonal
331 antibody against the conserved leucine-zipper region of human BATF3 (1:100, v/v~~,~~
332 ~~Merck, Cat. No. AB1007~~) for 60 min at room temperature. The sections were then
333 washed twice with washing buffer (Dako), ~~blocked~~ incubated with blocking solution

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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. A~~n a~~ntibody 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

al., 2001). 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 ~~It must be noted that the~~ polyclonal antibody against the conserved leucine-zipper region of human BATF3 was used, and that it~~used~~ 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

h~~a~~aematopoietic cells reside and adjacent to melano-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 rIFN~~s~~ but not by rF~~f~~lagellin, 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 rF~~f~~lagellin, polyI:C and R848, with ~~although~~ weaker induction ~~was~~ seen for BATF3a with~~by~~ rF~~f~~lagellin 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 F~~f~~lagellin (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~~but~~ both paralogues can be~~were~~ up-regulated (at least to some extent) in cultured cells by PAMPs and

484 cytokines, and in vivo by infection. Their potential role in fish immune responses is
485 discussed below.

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

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

508 Expression modulation of trout BATF3a and BATF3b was examined in primary
509 monocytes/macrophages isolated from head kidney and a spleen
510 monocyte/macrophage cell line (RTS-11). In the HK monocytes/macrophages,
511 BATF3a could be induced by all the stimuli used. Notably, stimulation with rIFNγ
512 resulted in the largest increase of BATF3a expression (55-fold increase). In contrast to
513 the HK monocytes/macrophages, only small changes in BATF3a and BAT3b

514 expression were seenwas largely unaltered in the RTS-11 cells, where relatively small
515 increases were seen after stimulation with LPS, Flagellin, poly I:C, R848 and rIFNγ,
516 with modulation of BATF3b mainly seen at the earlier time (6 h) post-stimulaiton. It
517 should be noted that the zinc finger transcription factors ZBTB46 and
518 DC-SCRIPT/ZNF366 known to be involved in activation of antigen presenting cells,
519 were down-regulated in primary HK monocytes/macrophages by rIFNγ, highlighting
520 the central roles of rIFNγ in mediating antigen presentation in fish (Zou et al., 2005;
521 Wang et al., 20186e; Wiegertjes et al., 2016; Zou et al., 2005).

522 IL-4 in combination with granulocyte macrophage colony stimulating factor
523 (GM-CSF) is commonly used for in vitro culture of dendritic cells in humans and
524 mice (Dauer et al., 2003). Two IL-4/13 homologues (IL-4/13A and IL-4/13B) have
525 been identified in rainbow trout but GM-CSF is absent in teleost fish (Zou and
526 Secombes, 2011; Wang et al., 2016c; Zou and Secombes, 2011d). Trout rIL-4/13A can
527 enhance HK monocyte/macrophage differentiation into CLEC4T+ cells (Johansson et
528 al., 2016). These cells express a remarkably high level of MHC II molecules and
529 moderate levels of the macrophage colony stimulating factor receptor (MCSFR) and
530 CD83, and display a distinct morphology when stimulated with bacterial and viral
531 PAMPs in association with inducible expression of ZBTB46 (Wang et al., 20186e). In
532 such cells, cultured with rIL-4/13A for 7 days, the transcript levels of BATF3a and
533 BATF3b remained largely unchanged (7-10 x 10^{-4} of BATF3a relative to EF-1α, 1-3 x
534 10^{-4} of BATF3b relative to EF-1α) (Figs. 5 and 8), but further stimulation, in
535 particular with viral PAMPs, enhanced expression several fold.

536 The in vivo studies demonstrated that the BATF3 paralogues are also modulated
537 during infection, in this case by bacterial and viral diseases. Relatively high increases
538 in transcript level were seen in the target organs; spleen in the case of Yersiniosis and
539 kidney in the case of IHNV. The kinetics of induction were was also studied duringin
540 the case of IHNV, where maximal increases were seen several days post-infection.
541 Taken together, the present findings suggest that BATF3 genes may have both
542 homeostatic and inducible functions within the immune system of fish, potentially in
543 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

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 n~~N~~eighbour-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 ~~I~~immunohistochemical staining (B). Twenty uL of an IPTG-induced overnight culture of *E. coli* BL21 (DE3) cells transformed with pHISTEV-BATF3a (Lane 1) or pHISTEV-BATF3b~~a~~ (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

604 24 h (A-D, B) with LPS (20 μg/mL), rFlagellin (20 ng/mL), polyI:C (50 μg/mL),
605 R848 (10 μg/mL), PHA (10 μg/mL), rIFN2 (type I) (20 ng/mL), rIFNγ (20 ng/mL) or
606 PBS (control). The gene expression levels were determined by real-time PCR and
607 normalized to that of EF-1α. The fold changes were calculated by comparing the
608 average expression level of each treatment group with that of the respective control
609 group. Bars indicate means +± SE of gene expression levels of cells from three flasks
610 for RTS-11 cells (n=3) or four fish for the primary HK monocytes/macrophages (n=4).
611 * = $p \leq 0.05$, ** = $p \leq 0.01$.
612 Fig. 8. Expression modulation of BATF3 in trout HK monocytes/macrophages
613 cultured with rIL-4/13A. The primary adherent HK leucocytes were isolated and
614 cultured in the presence of 200 ng/mL rIL-4/13A for 7 days. The cells were then
615 stimulated for 24 h with LPS (20 μg/mL), rFlagellin (20 ng/mL), polyI:C (50 μg/mL),
616 R848 (10 μg/mL), or PBS (control). The gene expression levels were determined by
617 real-time PCR and normalized to that of EF-1α. The fold changes were calculated by
618 comparing the average expression level of each treatment group with that of the
619 respective control group. Bars indicate means +± SE of gene expression levels of cells
620 from four flasks. * = $p \leq 0.05$, ** = $p \leq 0.01$. A, expression of BATF3a and BATF3b
621 in HK monocytes/macrophages cultured with rIL-4/13A for 7 days; B, fold changes of
622 expression of BATF3a and BATF3b after stimulation.
623 Fig. 9. Expression of BATF3 in rainbow trout after infection of *Y. ruckeri*. Fish were
624 challenged by intraperitoneal injection with a pathogenic strain (MT3072) of *Y.*
625 *ruckeri* (5×10^5 cfu per fish) or PBS. Gills, spleen and intestine were collected at 24 h
626 post-challenge and real-time PCR wasere performed to determine BATF3 expression.
627 The expression levels of BATF3a and BATF3b were normalized to that of EF-1α and
628 fold changes calculated by comparing the expression level of challenged fish with that
629 of the respective control fish (defined as 1). Bars indicate means +± SE of tissues
630 from three fish. * = $p \leq 0.05$.
631 Fig. 10. Expression of BATF3, Mx1 and IHNV N in rainbow trout juveniles (~3 g)
632 after infection with IHNV. The fish were injected intraperitoneally with 50 μL of L-15
633 medium containing 1×10^5 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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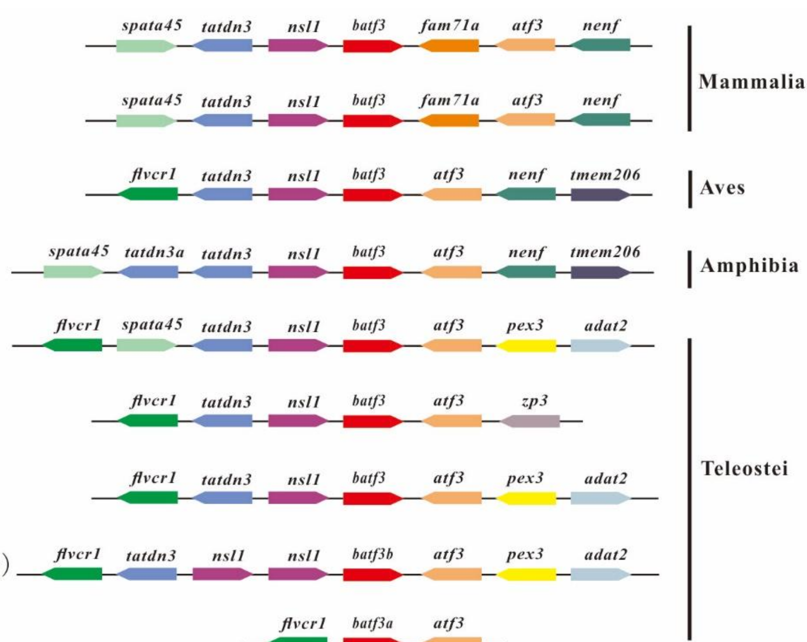
Figure

Fig. 1



Human (*Homo sapiens*)
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Mouse (*Mus musculus*)
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Chicken (*Gallus gallus*)
Chromosome 3:21114069-21291025

Frog (*Xenopus tropicalis*)
NW_004668237.1:112303290-112041079

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

Cave fish (*Astyanax mexicanus*)
Chromosome KB882090.1: 3896478-4004315

Amazon molly (*Poecilia formosa*)
Chromosome KI519970.1: 471807-538412

Rainbow trout (*Oncorhynchus mykiss*)
Scaffold_324 (CCAF010027628) : 802893-880777

Scaffold_1368 (CCAF010060656) : 138804-175945

Fig. 2

A

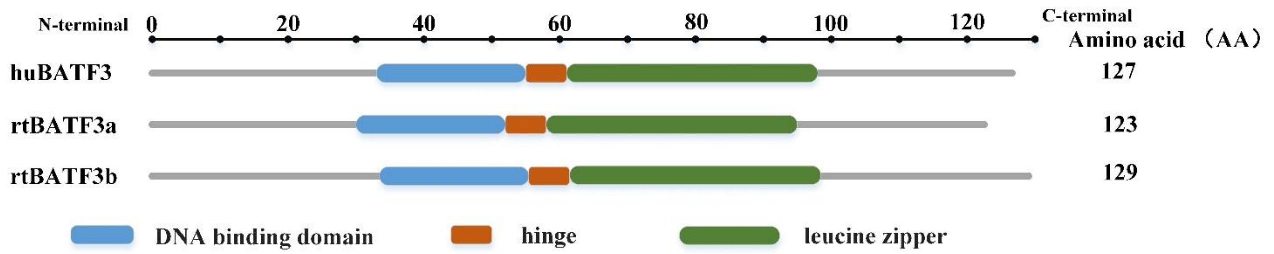
```
                                                                                  DB               H       LZ
Homo_sapiens                  -----MSQGLPAAGSVLQRSVAAPGNQPQPQPQQ---QSPEDDD--RKVRRREKNRVAAQRSRKKQTQKADKLHEEYESLEQEN 74
Mus_musculus                  -----MSQGPPAV-SVLQRSVDAPGNQPQ---------SPKDDD--RKVRRREKNRVAAQRSRKKQTQKADKLHEEHESLEQEN 67
Chrysemys_picta_bellii        -----MSLGVPASGSVLQRSASSDGNQPQ---------SPEEDD--RKIRRREKNRVAAQRSRKKQTQKADKLHEEYECLEQEN 68
Gallus_gallus                 MPRPHSNEPRLALLPILRRSCRFGKPMSHVFRGQECAGSHEEDD--KKVRRREKNRVAAQRSRKKQTQKADKLHEEYESLEQEN 82
Xenopus_tropicalis            -----MSERSPSASGTFQRSSAHNSSGSEADALS---HSSDTSD--RKVRRREKNRVAAQRSRKKQTQKADKLHEEYECLEQEN 74
Salmo_salar_BATF3a            -----------------MRVW-HSVPLLQ--------SSGDEDD-DWRLKRRENNRVAAQKNRKRQTQRADELHKAYECLDQKN 57
Oncorhynchus_mykiss_BATF3a    -----MSD--CDISSSFLQINDQSSFMLQRCE-----SSGDEDD-GWRHKRRENNRMAAQKSRNRQTQRADELHKAYECLDQKN 61
Salmo_salar_BATF3b            -----MSD--SDISGSFLHSKNQNMLLLERCELQ---SSGDDGDEDKRLKRREKNRVAAKNSRKKQTQRADELHEAYECLEQKN 74
Oncorhynchus_mykiss_BATF3b    -----MSD--SDISGSFLHSKNQNMLLLEICELQ---SSGDDGDEDRRLKRREKNRVAAKNSRKKQTQRADELHEAYECLEQKN 74
Oreochromis_niloticus         -----MSD--SGFS---CQSQQNNISTNQLCEGW---ECSEDEG--RRMKRREKNRVAAQKSRKRQTQRADLLHEACELLEQRN 69
Danio_rerio                   -----MSL--FSASSNFSRSDAPALRLYRQSE------SSDDDD--KRVKRREKNRVAAQRSRKRQTQRADELHEAYECLEQEN 69
                                                    :         .         . .  .   : :***:**:**:..*::***:** **:  * *:*.*

                                    LZ
Homo_sapiens                  TMLRREIGKLTEELKHLTEALKEHEKMCPLLLCPMNFVPVPP---RPDPVAGCLPR 127
Mus_musculus                  SVLRREISKLKEELRHLSEVLKEHEKMCPLLLCPMNFVQL-----RSDPVASCLPR 118
Chrysemys_picta_bellii        TSLKREIGKLTDEMKHLSEVLKDHEKICPLLHCSMNFVTIP----RPDALTSCLPR 120
Gallus_gallus                 TSLKKEIGKLTDEMKHLSEVLKDHEKICPLLHCTMNFVTIP----RPDALSSCLPR 134
Xenopus_tropicalis            SSLKKEIGKLTDELKHLSQILKDHEQICPFLHCPVNYVTVPR---VTDAVPGCLPR 127
Salmo_salar_BATF3a            RRLKKEVQFLSEEQRRLTEALKAHEPLCPIRHC-VPNLGS---GPRDVGVLSSLHR 109
Oncorhynchus_mykiss_BATF3a    RLLKKEVQFLSEEQMRLTEALKAHEPLCLIRHC-VPTLGS---GPRDVGVLSSLPR 123
Salmo_salar_BATF3b            RQLKKDVQFLSEEQRRLMEALKAHEPLCPIMHC-VANLGSGTLGPRDVGVPSCLPR 129
Oncorhynchus_mykiss_BATF3b    RQLKKDVQFLSEEQRRLTEALKAHEPLCPIMHC-VANLGSGTLGPRDVGVPSYLPR 129
Oreochromis_niloticus         RKLRREVDSLSEEQHLLTEALRAHEPFCPIMHCSFASSTSSTLQPENMAARSV--- 122
Danio_rerio                   SLLREEVQLLIEEQQRLTDALKAHEPLCRILTCGMTPITRST-GTVPPEFTSR--- 121
                                *:.::  * :*   * : *: ** :* :  * .                .
```

B

	O. mykiss BATF3a		*O. mykiss* BATF3b	
	Identity	**Similarity**	**Identity**	**Similarity**
H. sapiens	37.4%	51.8%	45.2%	60.7%
M. musculus	38.2%	56.5%	40.1%	55.5%
C. picta_bellii	39.2%	56.9%	43.8%	59.9%
G. gallus	36.4%	50.7%	38.8%	55.1%
X. tropicalis	38.2%	52.2%	45.0%	58.6%
***S. salar* BATF3a**	68.5%	70.8%	62.4%	70.7%
***O. mykiss* BATF3a**	---	---	69.0%	78.3%
***S. salar* BATF3b**	69.0%	78.3%	96.9%	97.7%
***O. mykiss* BATF3b**	69.0%	78.3%	---	---
O. niloticus	48.8%	60.5%	53.8%	66.9%
D. rerio	53.5%	64.3%	50.8%	62.9%

Fig. 3

A



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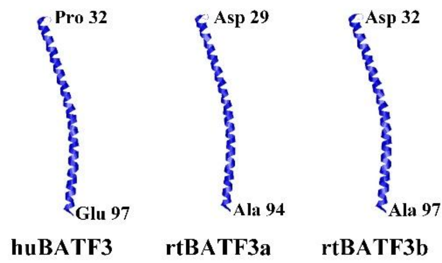


C

	N-terminal	bZIP	C-terminal
huBATF3 (AA)	32 (1-32)	65 (33-97)	30 (98-127)
rtBATF3a (AA)	29 (1-29)	65 (30-94)	29 (95-123)
rtBATF3b (AA)	32 (1-32)	65 (33-97)	32 (98-129)
Pair-wise identity/similarity			
rtBATF3a & huBATF3	5.9% / 9.8%	55.4% / 78.5%	36.4% / 42.4%
rtBATF3b & huBATF3	3.4% / 3.4%	61.5% / 83.1%	37.5% / 50.0%
rtBATF3a & rtBATF3b	53.1% / 65.6%	75.4% / 86.2%	71.9% / 71.9%

Fig. 4

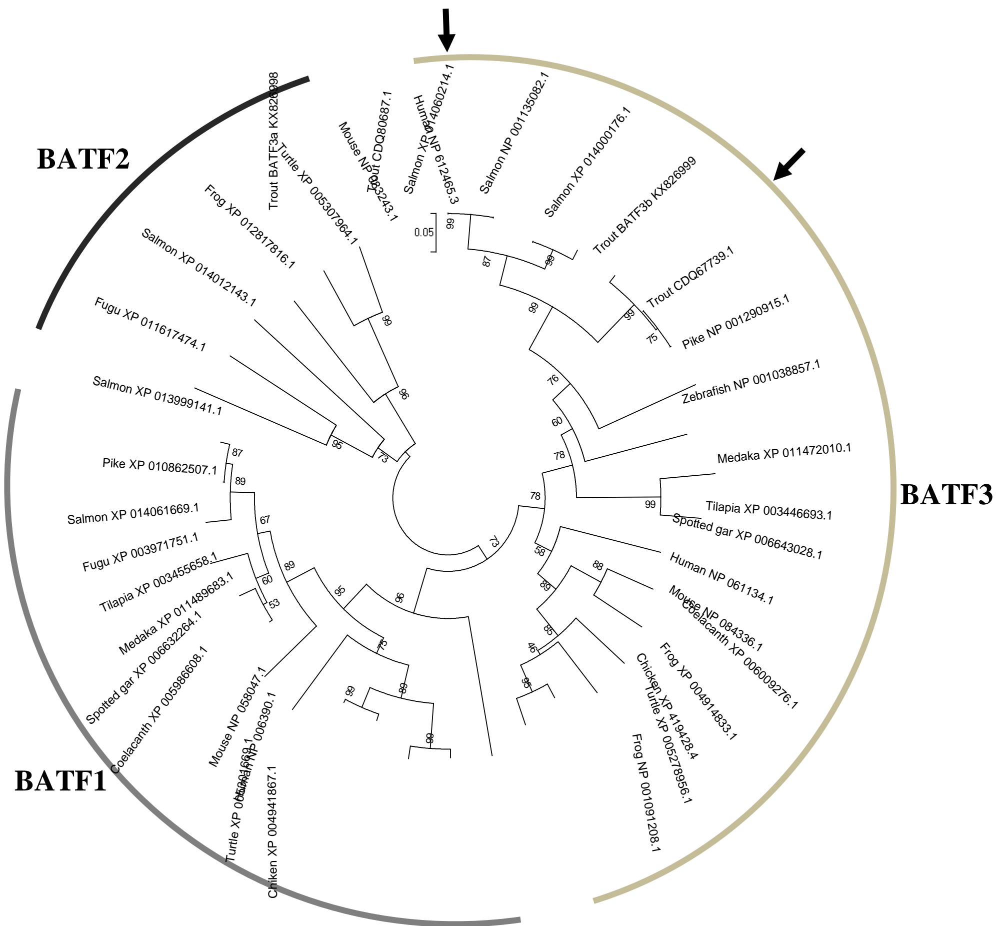


Fig. 5

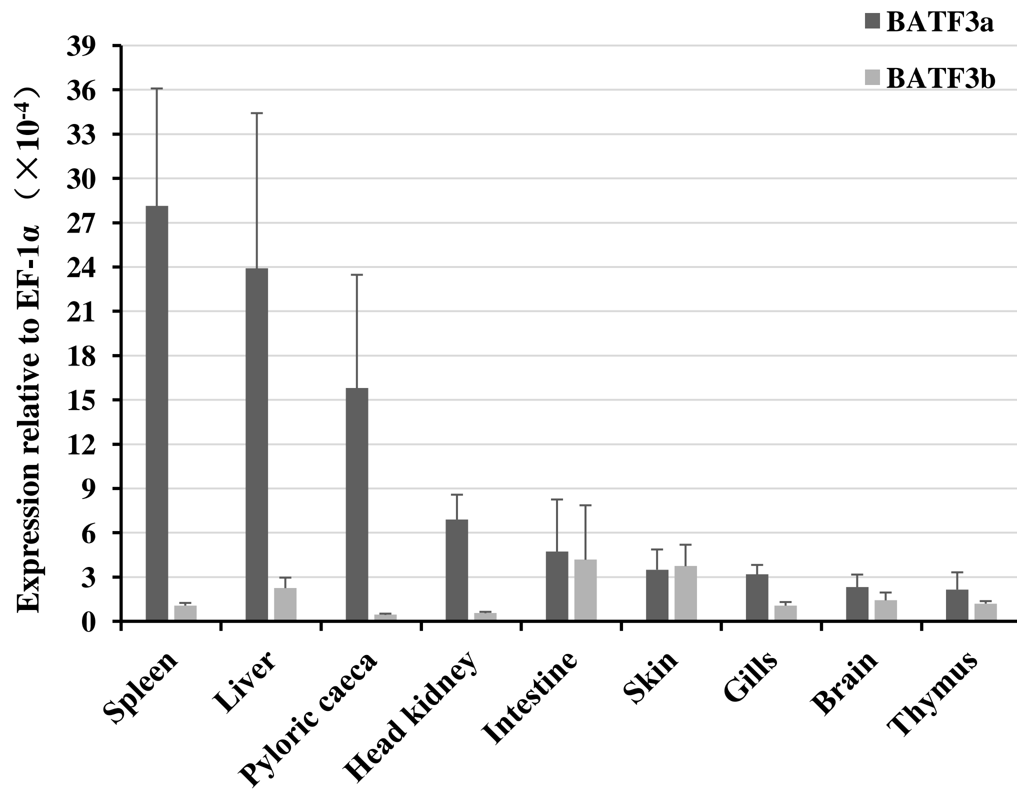
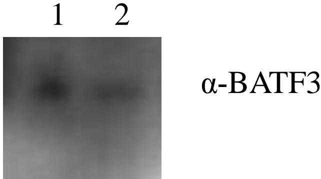


Fig. 6

A



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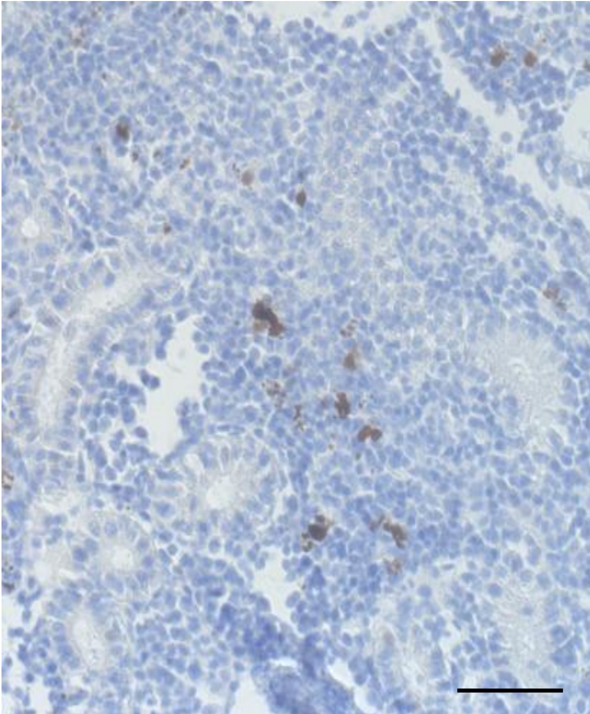


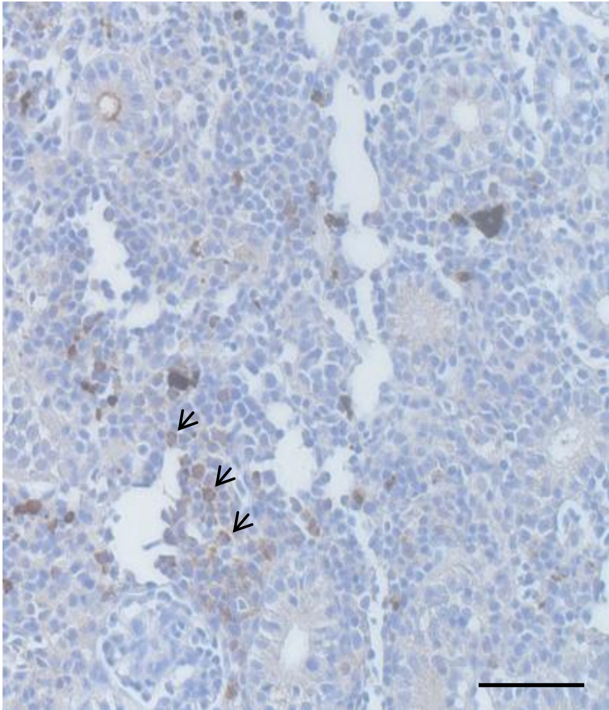
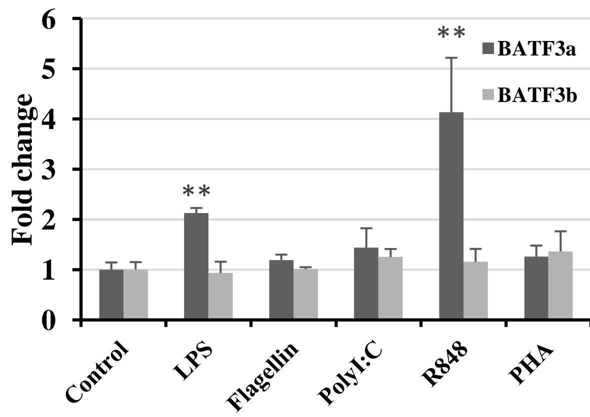


Fig. 7

A



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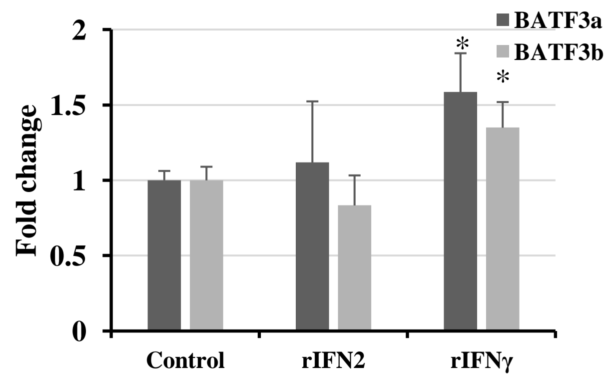

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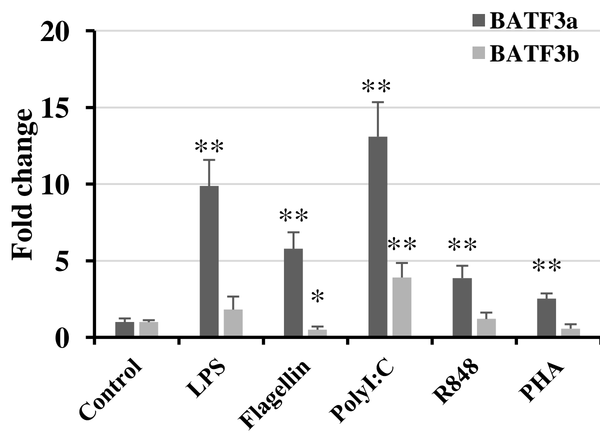

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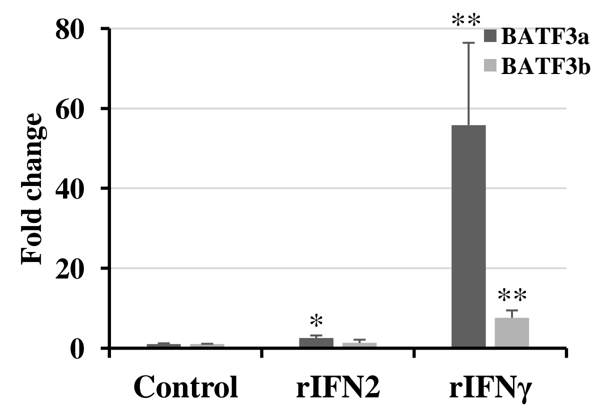


E

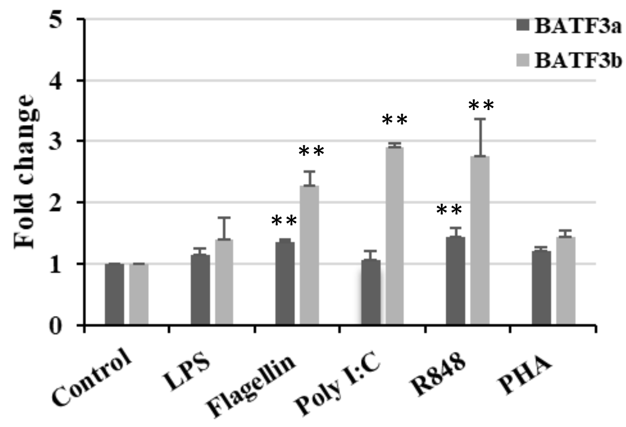
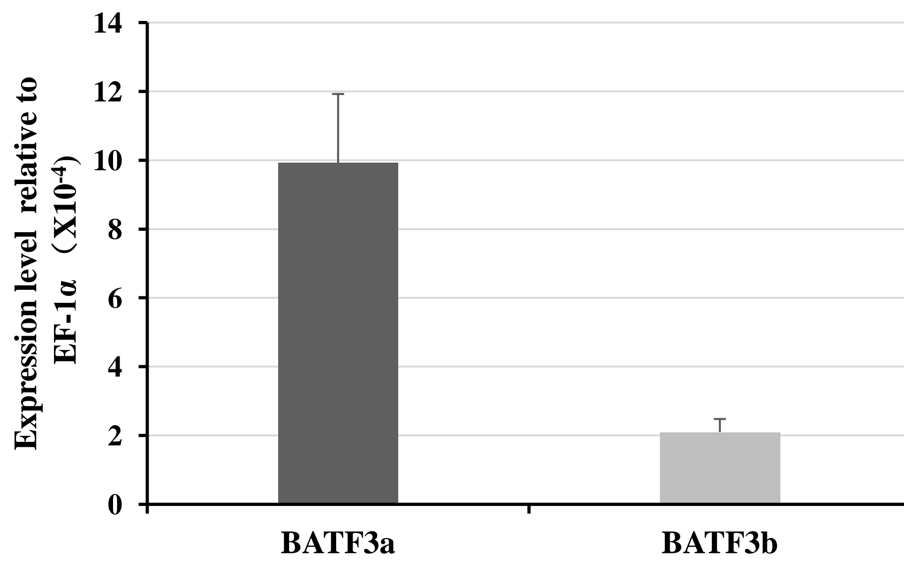


Fig. 8

A



B

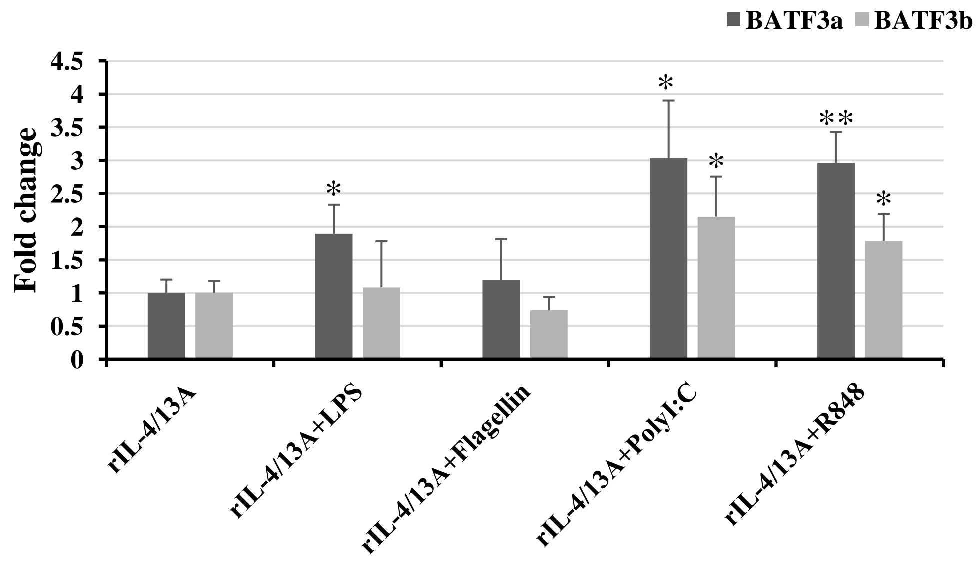

BATF3a
BATF3b
Fold change
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
**
*
rIL-4/13A
rIL-4/13A+LPS
rIL-4/13A+Flagellin
rIL-4/13A+PolyI:C
rIL-4/13A+R848

Fig. 9

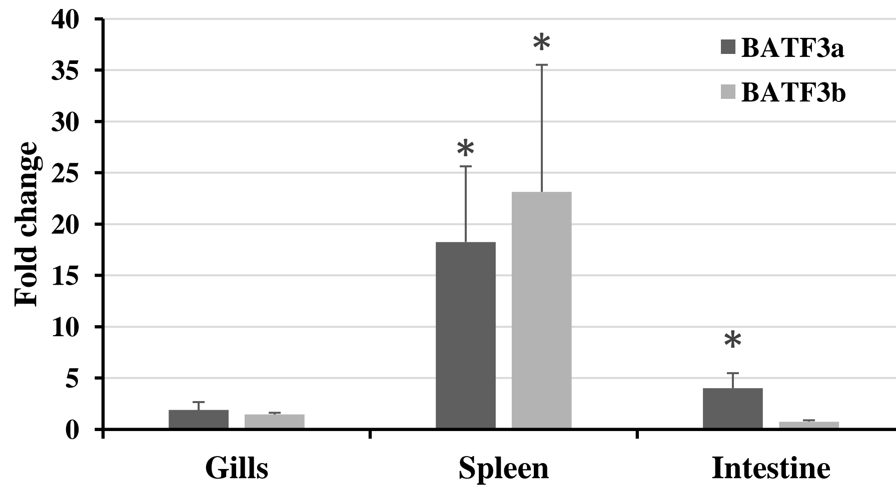
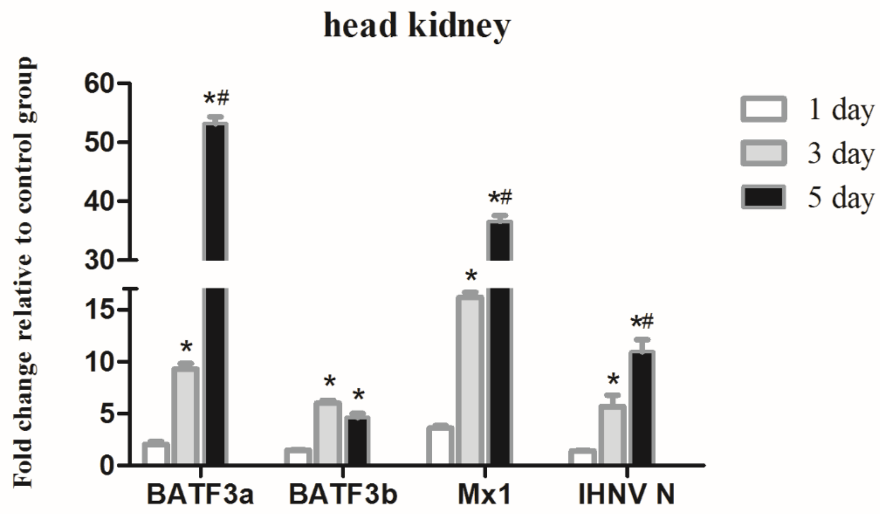


Fig. 10



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