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PII: S0145-305X(19)30223-X

DOI: https://doi.org/10.1016/j.dci.2019.103403

Article Number: 103403

Reference: DCI 103403

To appear in: *Developmental and Comparative Immunology*

Received Date: 5 May 2019

Revised Date: 23 May 2019

Accepted Date: 24 May 2019

Please cite this article as: Hong, S., Wang, T.-Y., Secombes, C.J., Wang, T., Different origins of paralogues of salmonid TNR1 and TNFR2: Characterisation and expression analysis of four TNF receptor genes in rainbow trout *Oncorhynchus mykiss*, *Developmental and Comparative Immunology* (2019), doi: https://doi.org/10.1016/j.dci.2019.103403.

Different origins of paralogues of salmonid TNR1 and TNFR2: characterisation and expression analysis of four TNF receptor genes in rainbow trout *Oncorhynchus mykiss*

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Running title: Rainbow trout TNF receptors

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The nucleotide sequence data will appear in the EMBL/DDBJ/GenBank nucleotide sequence database under the following accession numbers: HE717002 (TNFR1a), HE717003 (TNFR1b), HE717004 (TNFR2a) and HE717005 (TNFR2b).

Abstract

Mammalian TNFR1 and TNFR2 bind TNFα and TNFβ, and provide key communication signals to a variety of cell types during development and immune responses that are crucial for cell survival, proliferation and apoptosis. In teleost fish TNFβ is absent but TNFα has been expanded by the third whole genome duplication (3R WGD) and again by a 4R WGD in some lineages, leading to the four TNFα paralogues known in salmonids. Two paralogues for each of TNFR1 and TNFR2 have been cloned in rainbow trout in this study and are present in other salmonid genomes. Whilst the TNFR2 paralogues were generated via the 4R salmonid WGD, the TNFR1 paralogues arose from a local en bloc duplication. Functional diversification of TNFR paralogues was evidenced by differential gene expression and modulation, upstream ATGs affecting translation, ATTTA motifs in the 3'-UTR regulating mRNA stability, and post-translational modification by N-glycosylation. Trout TNFR are highly expressed in immune tissues/organs, and other tissues, in a gene- and tissue-specific manner. Furthermore, their expression is differentially modulated by PAMPs and cytokines in a cell type- and stimulant-specific manner. Such findings suggest an important role of the TNF/TNFR axis in the immune response and other physiological processes in fish.

Key words

Rainbow trout, TNFR1, TNFR2, paralogues, en bloc duplication, gene expression

1. Introduction

Tumor necrosis factor (TNF) receptor-1 (TNFR1) and TNFR2 are members of the TNF receptor superfamily (TNFRSF) that bind both TNFα and TNFβ, which belong to TNF superfamily (TNFSF). The TNFSF and TNFRSF provide key communication signals between a variety of cell types during development and immune responses, and have been implicated in many inflammatory and autoimmune diseases (Dostert et al., 2019). In humans, 19 TNFSF ligands and 29 TNFRSF receptors have been characterised to date (Collette et al., 2003).

The TNFSF members, such as TNFα (aka "TNFSF2"), are type II transmembrane (TM) proteins but can be secreted upon proteolytic cleavage by metalloproteases or furin proteases (Magis et al., 2010). All family members possess a TNF homology domain (THD) in the C terminal extracellular region that assemble into a non-covalent trimer essential for signalling by TNFSF members (Banner et al., 1993). Both membrane and soluble TNFα are bioactive through binding to TNFR1 and TNFR2 and play an important role in immune responses, inflammation, cell proliferation, differentiation, necrosis and apoptosis. TNFβ (aka "Lymphotoxin-α or LTα" and "TNFSF1") is the closest family member of TNFα in mammals. It can be secreted as a soluble homotrimer and binds to TNFR1 and TNFR2 with high affinity (Koroleva et al., 2018).

The TNFRSF receptors, such as TNFR1 (aka "TNFRSF1A") and TNFR2 (aka "TNFRSF1B") are type I TM receptors. The common feature of TNFRSF is the presence of relatively short (30–40 residues) cysteine-rich domains (CRD) located in the ectodomain, which are involved in interaction with the THD of TNFSF ligands (Dostert et al., 2019). The extracellular regions of TNFR1 and TNFR2 are structurally highly homologous and include four CRDs, each of which contains six cysteines. There is no significant homology in the intracellular region between TNFR1 and TNFR2, indicating that these receptors activate distinct signaling pathways (Puimege et al., 2014). TNFR1 contains a death domain (DD) in the cytoplasmic tail that recruits the TNFR1-associated DD protein (TRADD) and promotes cell death and inflammation. In contrast, TNFR2 does not have an intracellular DD and instead recruits the TNFR-associated factor (TRAF) 1 and TRAF2 proteins and favours cellular survival and tissue regeneration (Holbrook et al., 2019). Although both TNFα and TNFβ can bind and signal through TNFR1 and TNFR2, knock-out mouse mutants for each ligand have different phenotypes, partially due to differential expression of the ligands (Etemadi et al., 2013). TNFα is mainly produced by activated monocytes/macrophages but can also be produced by mast cells, T and B lymphocytes, natural killer (NK) cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts and osteoclasts, whereas TNFβ is secreted by activated T cells and resting B cells (Bradley, 2008).

The receptors of TNF are also differentially expressed and modulated. TNFR1 is ubiquitously expressed on nearly all cells in the body and the promoter of TNFR1 is active constitutively. The expression of TNFR2, on the other hand, is inducible and expressed exclusively in immune cells, endothelial cells and some neuronal populations (Puimege et al., 2014; Yang et al., 2018). The expression levels of TNFR proteins can be regulated by cytokines, such as interferons (IFN) and TNF itself (Aggarwal et al., 1985; Tsujimoto et al., 1986; Bloksma et al., 1983). Consequently, the activation from TNFR1 is responsible for a large number of inflammatory responses classically attributed to TNFα, although TNFR2 signalling has been reported to be important for proliferation of lymphoid cells and may be the preferential receptor for membrane TNFα (Grell et al., 1995).

The TNFSF/TNFRSF have a deep evolutionary origin that precedes the appearance of vertebrates and the adaptive immune system (Wiens and Glenney, 2011). They have been discovered in invertebrates including porifera (Pozzolini et al., 2016), molluscs (De Zoysa et al., 2009), crustaceans (Mekata et al. 2010; Wang et al. 2012) and insects (Kauppila et al., 2003), and expanded in vertebrates (Quistad and Traylor-Knowles, 2016). Genes coding for TNFSF members have also been studied in various fish species (Glenney and Wiens, 2007). TNFα was one of the first cytokines characterized within teleosts, initially isolated from Japanese flounder *Paralichthys olivaceus* (Hirono et al., 2000) and rainbow trout *Oncorhynchus mykiss* (Laing et al., 2001). Since then, it has been identified in a wide range of fish species. However, no orthologues of mammalian TNFβ has been identified in teleosts (Secombes et al., 2016; Maeda et al., 2018). Interestingly, two TNFα genes have been found in many teleost fish, eg. in bluefin tuna *Thunnus orientalis* (Kadowaki et al., 2009), orange-spotted grouper *Epinephelus coioides* (Lam et al., 2011), zebrafish *Danio rerio* and medaka *Oryzias latipes* (Kinoshita et al., 2014), goldfish *Carassius carassius* (Kajungiro et al.,2015) and meagre *Argyrosomus regius* (Milne et al., 2017), with even more TNFα genes identified in common carp *Cyprinus carpio* L. (Savan and Sakai, 2004) and salmonids (Hong et al., 2013). The paralogues reside in syntenically conserved regions on different chromosomes indicating they arose from WGD events known to have happened at the base of the teleosts (3R WGD) and again in the cyprinid and salmonid lineages (4R WGD) (Kinoshita et al., 2014; Berthelot et al., 2014; Xu et al., 2014).

In comparison, the receptors for TNFSF are less known in fish. Only a single TNFR1 and TNFR2 have been isolated in species such as Japanese flounder, goldfish and zebrafish (Park et al., 2003; Eimon et al., 2006; Grayfer and Belosevic, 2009). The Japanese flounder TNFR1 and TNFR2 share 35-40% identities to their mammalian counterparts (Park et al., 2003). The flounder TNFR1 was constitutively expressed in most organs whilst the TNFR2 gene was constitutively expressed in only immune organs (kidney, spleen and gills). In peripheral blood lymphocytes flounder TNFR1 and TNFR2 were differentially modulated by pathogen associated molecular patterns (PAMPs) and

activated by concanavalin A and phorbol myristate acetate (Park et al., 2003). In goldfish monocytes IFNγ upregulated the expression of both TNFR1 and TNFR2, whereas TNFα up-regulated TNFR2 but down-regulated TNFR1 (Grayfer and Belosevic, 2009). Whilst the bioactivity of TNFα has been described in rainbow trout (Hong et al., 2013), the receptors for TNFα have still to be characterised in salmonids.

In this study, two paralogues sharing high aa identity for TNFR1 and two for TNFR2 have been characterised in rainbow trout. Whilst the TNFR2 paralogues arose from the salmonid 4R WGD, the TNFR1 paralogues were generated by a local *en bloc* duplication. Their expression was comparatively examined by real-time PCR and it was found that the paralogues are differentially expressed *in vivo* in tissues from healthy fish and *in vitro* in cell lines stimulated with PAMPs (LPS and poly I:C) and recombinant cytokines (IFNγ and TNFα).

2. Methods and Materials

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*), weighing approximately 100 g, were purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, UK) and maintained in 1-m-diameter aerated fibreglass tanks supplied with a continuous flow of recirculating freshwater at 15 ± 1 °C. Fish were fed twice daily on standard commercial pellets (EWOS), and maintained as described previously (Wangkahart et al., 2019).

2.2 Cloning of trout TNF receptors.

The cloning of TNFR was performed in 2013 when no salmonid genomic resource was available. Blast (the basic local alignment search tool, Altschul et al., 1990) search was performed at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using mammalian TNFR1 and TNFR2 protein sequences, and resulted in the identification of candidate ESTs for TNFR1A (EZ763921), TNFR1B (EZ776440), TNFR2A (EZ832094) and TNFR2B (BX870761). Primers (Table 1) were designed within the 5'-untranslated region (UTR) of these ESTs and used for 3'-RACE as described previously (Wang and Secombes, 2003; Wang et al., 2008), using 3'-RACE-ready cDNA samples prepared from head kidney macrophages. The cloning, sequencing and protein sequence analysis was as described previously (Hong et al., 2013; Wang et al., 2018). The programs used included Clustal Omega (Sievers et al., 2011) for multiple sequence alignment, MatGAT program (V2.02, Campanella et al., 2003) for global sequence comparisons, SMART7 (Letunic et al., 2012) for domain prediction, MEGA7 (Kumar et al., 2016) for phylogenetic tree analysis and Genomicus (Louis et al., 2013) for synteny analysis.

2.3 RT-qPCR analysis of gene expression

The primers (Table 1) for real time PCR quantification of gene expression were designed so that at least one primer crossed an intron to prevent amplification of genomic DNA. A serially diluted common reference containing equal molar amounts of purified PCR products of trout TNFR1 and TNFR2 genes and the house-keeping gene EF-1α was used for quantification throughout. The real-time PCR quantification was as described previously, performed using a Lightcycler 480 system (Roche) (Wang et al., 2011a).

2.4 Tissue distribution of trout TNFR1 and TNFR2 transcripts

Six healthy rainbow trout (~ 100 g) were killed and seventeen tissues (blood, gills, thymus, scales,

skin, muscle, adipose tissue, liver, spleen, gonad, head kidney, caudal kidney, intestine, heart, tail fins, adipose fin, and brain) were collected and homogenised in TRI reagent (Sigma, UK). The RNA preparation and cDNA synthesis were as described previously (Wang et al., 2011a). The expression level of each gene in different tissues was normalized to the expression of EF-1α and expressed as arbitrary units.

2.5. Modulation of expression of TNFR1 and 2 in four trout cell lines.

Four trout cell lines, a mononuclear/macrophage-like cell line RTS-11 from spleen (Ganassin and Bols, 1998), an epithelial cell line from liver (Lee et al., 1993), and fibroid cell lines RTG-2 from gonad (Wolf and Quimby, 1962) and RTGill from gills (Schirmer et al., 1998) were used in this study. The cell culture conditions were as describe previously (Wang et al. 2011b). All cells were passaged 1 day before stimulation in L-15 medium (Invitrogen) supplemented with 10% FCS and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin) at a concentration of 3-5x10^5 cells/ml. The cells were stimulated by direct addition of stimulants that were dissolved in cell culture medium. Three stimulants, *E. coli* LPS (25 µg/ml, from strain 055:B5, Sigma), polyinosinic:polycytidylic acid (poly I:C, 50 µg/ml, Sigma), recombinant interferon (IFN)-γ (20 ng/ml, Wang et al., 2011b) or medium alone as control, were used and the cells incubated for 4, 8 and 24 h. The concentrations chosen for each stimulant were deemed optimal from previous studies (Wang et al. 2011b). RTS-11 cells were also stimulated with 10 ng/ml of recombinant TNFα3 (Hong et al., 2013) for 4, 8, 24, 48 and 72 h or storage buffer as a control. Four replicates (four flasks of cells) were used for each treatment, with the treatments terminated by dissolving the cells in TRI reagent (Sigma). The real-time RT-qPCR quantification of gene expression was as above. The fold change was calculated as the average expression level of stimulated samples divided by that of the time matched controls.

2.7. Statistical analysis

Real-time PCR data were analysed using the SPSS Statistics package 24.0 (SPSS Inc., Chicago, Illinois) as described previously (Wang et al., 2011a). One way-analysis of variance (ANOVA) and the LSD post hoc test were used to analyze expression data in Figs. 8-10, with $P < 0.05$ between treatment and control groups considered significant. Since tissue expression consisted of sample sets from six individual fish, a Paired-Sample T-test was applied (Fig. 6).

3. Results

3.1 Cloning and sequence analysis of trout TNFR1 and TNFR2 paralogues

The cDNA sequences of the two TNFR1 paralogues, TNFR1a and TNFR1b, are 3072 and 2688 bp and encode for 403 and 397 aa, respectively (**Figs. S1-2, Table 2**). There are two upstream ATGs in the 5'-UTR, and mRNA instability motifs (ATTTA, 1 for TNFR1a and 4 for TNFR1b) in the 3'-UTR suggesting that they may be subject to post-transcriptional and translational regulation. The predicted aa sequences of both cDNA sequences have a signal peptide, an extracellular region containing 4 CRDs each with six conserved cysteine residues, a TM domain, and an intracellular DD domain. All these characteristics are well conserved across TNFR1 molecules from other fish species (Atlantic salmon, northern pike, channel catfish and Japanese flounder) and mammals (humans and mice) (**Fig. 1**). There are two potential N-glycosylation sites present in trout TNFR1a and only one in TNFR1b that were conserved in salmon counterparts. The first site was found in CRD1 of salmonids TNFR1s that was also found in mammalian TNFR1s. The other site of salmonid TNFR1 was in CRD4 with an N-glycosylation site also found in pike and catfish in the same domain (**Fig. 1**). The major difference between fish and mammalian TNFR1 was an insertion of low complexity region rich in P/S/T residues between the TM and DD domains (**Fig. 1**).

The cDNA sequences of the two trout TNFR2 paralogues, TNFR2a and TNFR2b, are 1814 and 2403 bp that encode for 455 and 463 aa, respectively (**Figs. S3-4, Table 2**). There are 2 ATTTA motifs in the 3'-UTR of TNFR2b but none in TNFR2a. The predicted aa sequences of both cDNA sequences have a signal peptide, an extracellular region containing 4 CRDs, a TM domain, and an intracellular domain. CRD1-3 possess six cysteine residues but CRD4 only four. All these characteristics are well conserved across TNFR2 molecules from other fish species and mammals (**Fig. 2**). There are three potential N-glycosylation sites present in trout TNFR2a and two in TNFR2b. The first site was found in CRD1 of trout TNFR2 that was conserved in salmon TNFR2b, as well as flounder and tilapia TNFR2. The other trout sites were in CRD4 with at least one N-glycosylation site also found in other vertebrate TNFR2 molecules in the same domain except salmon TNFR2b (**Fig. 2**).

The predicted aa sequences of the two trout TNFR1 paralogues share 80.2% aa identity (**Table 2**). The trout and salmon orthologues shared higher aa identities (ie. 85.5% for TNFR1a and 93.0% for TNFR1b) than paralogues between and within species (79.8-82.4%). All salmonid TNFR1 molecules shared similar aa identities/similarities to TNFR1 from other fish species and mammals (**Table S1**). The translation of the two trout TNFR2 paralogues share 63.6% aa identity (**Table 2**). The trout and salmon orthologues again shared higher aa identities (ie. 91.2% for TNFR2a and 87.2% for TNFR2b) than paralogues between and within species (59.6-64.4%). All salmonid TNFR2 molecules shared

similar aa identities/similarities to TNFR2 from other fish species and mammals (**Table S2**).

3.2 Bioinformatics analysis of TNFR1 and TNFR2

To confirm the membership of the trout genes cloned in this study in the TNFRSF, a phylogenetic tree was constructed using amino acid multiple alignments of vertebrate TNFR1, TNFR2, and other closely related tetrapod TNFRSF members (TNFR3, 5, 10 and 14) sharing similar domain structure (Magis et al., 2012). Trout TNFR1 and TNFR2 grouped with their counterparts from other fish species and tetrapods with high bootstrap support (99% for TNFR1 and 96% for TNFR2) and these clades were separated from other closely related TNFRSF members (**Fig. 3**), confirming their identities. The topology of salmonid TNFR1 and pike (a close relative of salmonids before the salmonid 4R WGD) TNFR1 represents a classical tree topology recapturing true species relationships after a WGD (Macqueen and Johnston, 2014). The salmonid and pike TNFR2 also form an independent clade but with pike TNFR2 set between salmonid TNFR2a and TNFR2b (**Fig. 3**). In both cases it is possible to suggest that the salmonid paralogues arose from the salmonid 4R WGD.

To further confirm their identities, we performed a synteny analysis with the Genomicus program using medaka TNFR1 and TNFR2 as references. Both vertebrate TNFR1 and TNFR2 loci were well conserved in medaka, tetraodon, zebrafish, chicken, humans and mice as shown in **Fig. S5**, suggesting a true orthologous relationship.

The pike and salmonid genomes are not well annotated in the current Genomicus database. Therefore, we performed a manual synteny analysis with information extracted from the updated genomic sequences from NCBI. Pike TNFR1 was found in linkage group (LG)20, and both trout TNFR1a and TNFR1b on Ch3 (**Fig. 4A**). Most of the genes in the pike TNFR1 locus were found present in trout with a block of genes (dedd2 to TNFR1) duplicated adjacently in trout Ch3. The duplicated blocs retained most gene present in pike and have an additional CD27 gene in both blocs (**Fig. 4A**), suggesting this gene was present before the en bloc duplication. Similarly, the salmon TNFR1a and TNFR1b were also found in the syntenic region on Ch2 (NC_027301), suggesting that the salmonid TNFR1 paralogues arose from a local en bloc duplication.

Pike TNFR2 was found in LG17, and trout TNFR2a and TNFR2b in Ch17 and Ch7, respectively. The TNFR2 loci on pike LG17, trout Ch17 and Ch7 were well conserved (**Fig. 4B**). Similarly, salmon TNFR2a and TNFR2b were also found in a syntenic region on Ch12 (NC_027311) and Ch22 (NC_027321), respectively. These syntenic relationships confirm that the salmonid TNFR2 paralogues indeed arose from the salmonid 4R WGD.

3.3 Gene organization analysis of TNFR1 and TNFR2

Both TNFR1 and TNFR2 in humans and chicken have a 10 exon/9 intron gene organization with all 10 exons coding, and identical intron phases (**Fig. 5**). Both trout TNFR1 paralogues, and flounder TNFR1 also have a 10 exon/9 intron structure. However, there is 1 non-coding exon in the 5'-UTR and only 9 coding exons (**Fig. 5A**). Fish TNFR1 genes has a large exon 8 that is equivalent to exons 7 and 8 in human and chicken TNFR1 possibly caused by intron insertion in tetrapods or intron lose in fish. The human TNFR1 gene has a large exon 9 that contributes to the insertion of the low complexity region between TM and DD domains in the TNFR1 multiple alignment (**Fig. 1**). Fish TNFR2 genes had a similar gene organization to that of tetrapods but again had one less coding exon. The coding region of the last exon in fish was large and equivalent to that of exons 9 and 10 in tetrapods, possibly caused by intron insertion in tetrapods or intron lose in fish (**Fig. 5B**).

3.4. Tissue distribution of the expression of trout TNFR1 and TNFR2 transcripts

Constitutive expression of the four TNF receptor genes was detectable in all seventeen tissues examined (**Fig. 6**). The highest expression of TNFR1a was in spleen and lowest in liver. The highest expression of TNFR1b was also found in spleen as well as heart, and the lowest in liver and tail fins (**Fig. 6**). The spleen, gills, brain, adipose tissue and tail fins expressed higher levels of TNFR1a than TNFR1b. In contrast, heart, muscle and blood expressed higher levels of TNFR1b than TNFR1a (**Fig. 6**).

The highest expression of TNFR2a was in spleen and heart, and the lowest in liver, tail fins and scales. The highest expression of TNFR2b was also found in spleen and heart, in addition to caudal kidney, and the lowest in blood. The two TNFR2 paralogues were differentially expression in all the tissues except for gills, brain, adipose tissue, scales and skin (**Fig. 6**).

Overall, trout TNF receptor genes were differentially expressed in a tissue-dependent manner, and the expression of TNFR1 genes was higher than that of TNFR2 genes in all tissues tested, by greater than ten-fold in most cases. The immune tissues, eg. spleen, gills and head kidney, are among those that highly expressed all the receptors. The high-level expression of TNFR2 paralogues in non-immune tissues/organs, including heart, caudal kidney, gonad and muscle was also noteworthy.

3.5. Differential expression of trout TNFR1 and TNFR2 paralogues in four cell lines

The TNF receptor gene were also found differentially expression in trout cell lines (**Fig. S6**). The expression of TNFR1 paralogues was higher than that of TNFR2 paralogues in all the four cell lines studied. The macrophage-like cell line RTS-11 expressed the highest levels of all the receptors (**Fig. S6**).

3.6. Modulation of the expression of TNFR1 and TNFR2 paralogues in four trout cell lines.

In epithelial cell lines RTL and RTGill, the expression of TNFR1 paralogues was high constitutively but was refractory to stimulation with LPS, poly I:C and IFNγ (**Fig. 7**). TNFR2a expression was up-regulated by IFNγ from 4 h to 24 h, and by polyI:C from 8 h to 24 h but was refractory to LPS. Meanwhile, TNFR2b expression was upregulated only by poly I:C in RTGill cells at 24 h, but down-regulated by IFNγ in RTL at 24 h (**Fig. 7**). In the fibroblast like RTG-2 cells, IFNγ up-regulated TNFR1a and TNFR1b expression at 4h and 8 h, and TNFR2a expression from 4 h to 24 h, but decreased TNFR2b expression at 24 h. Poly I:C also upregulated TNFR1a and TNFR1b expression at 8 h only, and TNFR2a expression at 8h and 24 h, but had no effects on TNFR2b expression. LPS moderately up-regulated TNFR2a expression at 4 h but had no effects on the expression of other TNF receptor genes in this cell line (**Fig. 7**). In RTS-11 cells, IFNγ rapidly up-regulated TNFR1a and TNFR1b expression at 4 h, and TNFR2a expression from 4h to 24 h, but decreased the expression of TNFR1 paralogues at 24 h, and had no effect on TNFR2b expression. Poly I:C up-regulated TNFR2b expression at 4 h only and had no effects on the expression of other genes. LPS increased the expression of TNFR1b and TNFR2b at 4 h only and had no effects on the expression of TNFR1a and TNFR2a (**Fig. 7**).

In conclusion, the expression of TNF receptor genes can be modulated in a cell line-, gene- and stimulant-dependent manner. TNFR1 paralogues are highly expressed constitutively but less responsive to stimulation (less than 10-fold increase). In contrast, TNFR2a expression was low constitutively but highly inducible. IFNγ is a potent stimulant of TNFR expression with rapid kinetics of induction compared to poly I:C. LPS only had minor effects on TNFR expression (**Fig. 7**).

3.7. Modulation of the expression of TNFR1 and TNFR2 paralogues by recombinant TNFα

RTS-11 cells express the highest level of TNFR1 and TNFR2 genes and are known to be responsive to TNFα stimulation (Hong et al., 2013). Therefore, we examined the receptor expression after TNFα treatment of RTS-11 cells over a time course from 4 h to 72 h. Significant up-regulation of gene expression was seen with TNFR1a from 8-48 h, TNFR1b at 4 h, 24 h and 48h, and TNFR2a from 4-48 h (**Fig.** 8). The up-regulated expression was absent by 72 h. TNFR2b expression was refractory at all time-points (**Fig.** 8).

4. Discussion

In this study, two highly identical paralogues of TNFR1 and TNFR2 have been cloned in rainbow trout. Their identities were confirmed in terms of the characteristic domain structure of the protein encoded, by phylogenetic tree analysis, and by conserved synteny. Many highly identical immune genes, eg. TNFα, IL-1β, IL-12 family members and SOCS gene family members, are present in salmonids that originate from the salmonid 4R WGD (Hong et al., 2013; Husain et al., 2014; Wang and Husain, 2014; Wang et al., 2019). This also appears to be the case for the two TNFR2 paralogues. However, to our surprise, the two trout TNFR1 paralogues arose from a local en bloc duplication that has also happened in other salmonids such as Atlantic salmon. The salmonid en bloc duplicated TNFR1 paralogues share higher identities at the protein level (eg. 80.2% in trout) compared to the 4R WGD originated TNFR2 paralogues (eg. 63.6% in trout). This reveals that both duplicated TNFR2 loci were retained after the 4R WGD but one copy of the duplicated TNFR1 locus was lost. The retained TNFR1 copy later duplicated en bloc in an ancestral salmonid. The mechanisms driving this en bloc gene duplication and retention is unknown but could be due to gene balance, whereby genes in the same complex pathway are preferentially co-retained after WGDs to avoid architectural disruption or metabolic imbalance (Pires and Conant, 2016). Two types of TNFα, the ligand binding TNFR1 and TNFR2, are present in 3R teleosts, and these were duplicated again by the salmonid 4R WGD and retained as four paralogues in salmonids (Hong et al., 2013; and unpublished data). Mammalian TNF (including TNFα and TNFβ) signal through TNFR1 to promote mainly inflammation and apoptosis, whilst TNFR2 signaling activates the pro-survival PI3K-Akt/PKB pathway and sustains regulatory T cell function (Pegoretti et al., 2018). Dysfunction of either TNF, TNFR1 or TNFR2 hampers immune defence or promotes inflammatory and autoimmune diseases (Puimege et al., 2014; Yang et al., 2018). The en bloc duplication of TNFR1 in salmonids may represent a kind of convergent evolutionary mechanism to produce multiple copies of TNFR1 to balance the multiple copies of TNFα and TNFR2 generated by WGD.

Gene duplication is a major driver of functional divergence. The duplicated genes are preserved through functional diversification: neofunctionalization, subfunctionalization, or both (Teufel et al., 2019). The functional divergence can be embodied by changes in the promoter that regulate the expression, or changes with effects on regulation at post-transcriptional, translational and post-translational levels, and changes in the protein sequence that directly affect its function. The regulation of mRNA stability is important for the control of gene expression. AU rich elements, such as AUUUA motifs (ATTTA in cDNA sequence) are important cis-acting sequences in the 3'-UTRs of mRNAs encoding cytokines and other transiently expressed genes that promote mRNA degradation

(Mino and Takeuchi, 2018). Four ATTTA motifs are present in the 3'-UTR of TNFR1b but only two in TNFR1a and TNFR2b, with none in TNFR2a. These differences may suggest that the TNFR1 and TNFR2 paralogues differ in post-transcriptional regulation.

Translation initiation is the rate-limiting step in mRNA translation and is central to translational regulation. Upstream ATG/open reading frames (uORFs) in the 5'-UTR are regulatory elements that modulate the translation initiation rate of the downstream ORF by sequestering ribosomes (Zhang et al., 2019). Two upstream ATG are present in TNFR1 paralogues and one in TNFR2b, suggesting that trout TNFR genes may subject to translational regulation.

N-Glycosylation is a co- and post-translational modification that is critical for the regulation of the biophysical properties and biological activities of diverse proteins (Zacchi et al., 2016). Mammalian TNFR1 can be modified by N-glycosylation that could facilitate its capability of binding to TNFα and signalling (Han et al., 2015). 1-3 potential N-glycosylation sites are found mainly present in CRD1 and CRD4 in different trout TNFR molecules. Although the implication of N-glycosylation remains to be determined in fish, the different potential of N-glycosylation of TNFR1 and TNFR2 paralogues may suggest divergence of post-translational regulation.

Another interesting finding in this study is the difference of gene organisation between tetrapod and teleost TNFR1 and TNFR2 orthologues. Teleost fish TNFR1 genes have an extra exon in the 5'UTR. In addition, both teleost TNFR1 and TNFR2 possess one fewer coding exon than their tetrapod orthologues and a large coding exon/region that equates to two exons in tetrapods. Whether this change was due to intron loss in teleosts or intron insertion in tetrapods is not possible to answer from this study. Exon insertion in the 5'-UTR may bring extra control elements such as upstream ATG/ORFs, as seen with the two trout TNFR1 paralogues, that may impacton translation regulation (Zhang et al., 2019). Introns provide selective advantages to eukaryotic cells, such as regulating gene expression, alternative splicing and nonsense-mediated decay, controlling mRNA transportation and chromatin assembly (Jo and Choi, 2015). Gene expression analysis of paralogues revealed that those with structural change showed large differences and a low correlation coefficient between paralogues (Wang et al., 2019). Thus, change of exon/intron gene organisation might have an impact on gene expression and function

At the transcript level, expression of four trout TNFR genes is detectable in all the tissues analysed (from healthy fish) and in cell lines, albeit at different levels, suggesting a ubiquitous nature of their expression in rainbow trout. The protein expression levels remain to be determined when isoform-specific antibodies are available in the future. The expression of TNFR1 paralogues is higher than that

of the TNFR2 paralogues in most tissues and cell lines, in accord with the ubiquitous expression of TNFR1 and more limited expression of TNFR2 seen in mammals (Yang et al., 2018). However, the paralogues are differentially expressed in a tissue- and cell line-specific manner, suggesting functional diversification.

The expression of TNFR1 and TNFR2 paralogues is high in immune organs, such as spleen, head kidney and gills in rainbow trout, as seen in Japanese flounder (Park et al., 2003), suggesting important roles in the fish immune response as in mammals (Puimege et al., 2014). Their expression is low in liver, especially for TNFR1 paralogues. Liver is an important immune organ involved in acute phase reactions, that under stress and infections produces large quantities of inflammatory substances including TNF (Khansari et al., 2019). The low levels of expression of TNFR1 paralogues in resident liver cells may prevent excessive TNFR1 signaling leading to inflammatory shock and apoptosis. The high level expression of TNFR1 and TNFR2 in muscle may reflect the direct action of TNFα on skeletal muscle shown in mammals (Li, 2003) and in rainbow trout (Vraskou et al., 2011). Another interesting observation is the high level expression of the TNFR2 paralogues in non-immune organs including heart, caudal kidney, muscle and gonad. TNF binding to TNFR2 activates NF-κB and PI3/Akt pathways that maintain survival and enhance proliferation (Yang et al., 2018), suggesting an important role of the TNF/TNFR2 axis in these organs.

Trout TNF receptors can be modulated in a gene-, cell line- and stimulant-dependent manner. In general, the expression of TNFR1 paralogues is higher constitutively but less responsive to stimulation with PAMPs and recombinant cytokines than TNFR2 paralogues, especially for TNFR2a, in the four cell lines tested. The cell type-dependent modulation of TNF receptor expression may be of relevance in terms of curbing inflammatory signals to keep a balance between survival and death signals. It is known that TFNα expression can be induced in these cell lines by LPS, poly I:C, IFNγ and TNFα, and is highly induced *in vivo* by bacterial and viral infection (Hong et al., 2013). The expression of TNFR1 paralogues was refractory to stimulation in the epithelial-like RTL and RTGill cells. In contrast, TNFR2a is highly induced by polyI:C and IFNγ. In RTG-2 and RTS-11 cells, TNFR1 paralogues can be up-regulated but to a lower extent and more transiently compared to TNFR2a. The preferential up-regulation of TNFR2 expression may enhance survival and proliferation to maintain an intact epithelium and help tissue-repair after an insult.

It seems that IFNγ is a potent inducer of trout TNF receptor expression, as seen in carp (Grayfer and Belosevic, 2009) and mammals (Aggarwal et al., 1985; Tsujimoto et al., 1986). Poly I:C can also up-regulate TNFR expression especially for TNFR2, but LPS has only minor effects. The poor responsiveness to LPS in trout cells may be due to the loss of TLR4 in the genome, as seen in most

fish species, and even when present (as in zebrafish) does not appear to bind LPS (Sepulcre et al., 2009). The expression of trout TNFR1a, TNFR1b and TNFR2a can be up-regulated by TNFα in RTS-11 cells but TNFR2b was refractory. Interestingly, goldfish TNFα up-regulates TNFR2 expression but down-regulates TNFR1 expression in monocytes (Grayfer and Belosevic, 2009). Whether this inconsistency is due to species-specific regulation or to cell type/developmental stage differences remains to be determined.

Conclusion: Two paralogues of TNFR1 and TNFR2 are present in salmonids. Whilst the TNFR2 paralogues were generated via the 4R salmonid WGD, the TNFR1 paralogues arose from a local en bloc duplication. Functional diversification of TNFR paralogues was evidenced by differential gene expression and modulation, upstream ATGs affecting translation, ATTTA motifs in the 3'-UTR regulating mRNA stability, and post-translational modification by N-glycosylation. Teleost and tetrapod TNFR1 and TNFR2 orthologues differ in exon/intron organization. Trout TNF receptors are highly expressed in immune tissues/organs, and in other tissues in a gene- and tissue-specific manner. Their expression can be differentially modulated by PAMPs and cytokines in a cell type- and stimulant-specific manner, suggesting an important role of the TNF/TNFR axis in the immune response and other physiological processes in fish.

5. Acknowledgements

SH was financially supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2017R1A2B2011167). T-YW was financially supported by the Ministry of Science and Technology, Taiwan (MOST 107-2917-I-564-019). TW received funding from the Marine Alliance for Science and Technology for Scotland (MASTS), a pooling initiative funded by the Scottish Funding Council (grant reference HR09011).

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Figure legend

Fig. 1. Multiple alignment of rainbow trout TNFR1 paralogues with TNFR1 molecules from other fish and mammalian species. The multiple alignment was produced using Clustal Omega, and conserved amino acids were shaded using BOXSHADE (V3.21). The signal peptide, the four cysteine rich domains (CRD1-4), the transmembrane domain and the death domain are indicated above the alignment. The aa sequence of the signal peptide and transmembrane domain are in green, and the conserved cysteine residues in the CRD domains are in red. Potential N-glycosylation sites in CRD1 and CRD4 are highlighted in blue.

Fig. 2. Multiple alignment of rainbow trout TNFR2 paralogues with TNFR2 molecules from other fish and mammalian species. The multiple alignment was produced using Clustal Omega, and conserved amino acids were shaded using BOXSHADE (V3.21). The signal peptide, the four cysteine rich domains (CRD1-4) and the transmembrane domain are indicated above the alignment. The aa sequence of the signal peptide and transmembrane domain are in green, and the conserved cysteine residues in the CRD domains are in red. Potential N-glycosylation sites in CRD1 and CRD4 are highlighted in blue.

Fig. 3 A unrooted phylogenetic tree of selected vertebrate TNFRSF members. The phylogenetic tree was constructed using amino acid multiple alignments of TNFR1, TNFR2, and other closely related TNFRSF members (TNFR3,5,10,14) from selected vertebrates, and the neighbour-joining method within the MEGA7.0 program. The evolutionary distances were computed using the JTT matrix-based method with all ambiguous positions removed for each sequence pair. Node values represent percent bootstrap confidence derived from 10,000 replications. The accession number for each sequence is given after the species and molecule names. The trout molecules cloned in this study are highlighted in red and grouping of TNFRSF members are indicated on the right.

Fig. 4. The TNFR1 (A) and TNFR2 (B) loci of rainbow trout and pike. The information was extracted from genomic DNA sequences at NCBI under accession numbers NC_025987 (pike TNFR1), NC035079 (trout TNFR1a and TNFR1b), NC_025984 (pike TNFR2), NC035093 (trout TNFR2a) and NC035083 (trout TNFR2b).

Fig. 5. Comparison of gene organisation of the TNFR1 (A) and TNFR2 (B) in rainbow trout, flounder, humans and chicken. Boxes represent exons, and lines between exons represent introns. The white and black boxes represent non-coding and amino acid (aa) coding regions, respectively. The sizes (bp) of each exon are numbered in the boxes. The gene organization of the rainbow trout TNFR1 and 2 genes was predicted using the Splign program based on the sequence information from Table 1 and Figs. S1–S4 in Supplementary Materials. The accession numbers for other genes are

XM_020093063/NW_017859661 (flounder TNFR1), AB080947/NW_017859683 (flounder TNFR2), ENSG00000067182 (human TNFR1), ENSG00000028137 (human TNFR2), ENSGALG00000039461 (chicken TNFR1) and ENSGALG00000034213 (chicken TNFR2). The TNFR protein domains (SP=signal peptide, TM=transmembrane domain) and corresponding coding exon regions are indicated.

Fig. 6. Transcript expression of rainbow trout TNFR1 and TNFR2 paralogues in tissues. The expression level was determined by RT-qPCR in 17 tissues from six healthy fish. The transcript level was calculated using a serial dilution of references that contained equal molar amounts of the probes for each gene, then normalized against the expression level of EF-1α and presented as the average + SEM. The connected bars indicate significant differences in expression levels of paralogues ($p < 0.05$, paired samples T test).

Fig. 7. **Modulation of the expression of TNFR1 (A, C, E and G) and 2 (B, D, F and H) paralogues in four cell lines by PAMPs and IFNγ.** Four trout cell lines, RTL, RTG-2, RTGill and RTS-11 cells were stimulated with LPS (25 μg/ml), poly I:C (25 μg/ml) and recombinant IFNγ (10 ng/ml) for 4, 8 and 24 h. The expression of TNF receptors was quantified as in Fig. 6, and presented as the mean (+SEM, N=4) fold change calculated by the average expression level of treated samples divided by that of time-matched controls. The relative significance of a LSD post hoc test after a significant one-way ANOVA between the stimulated and time-matched controls is shown above the bars as * $p \leq 0.05$, **$p \leq 0.01$ and *** $p \leq 0.001$.

Fig. 8. **Modulation of the expression of TNFR1 and TNFR2 paralogues in RTS-11 by TNFα.** RTS-11 cells were stimulated with 10 ng/ml recombinant TNFα3 for 4-72 h. The expression of TNF receptors was quantified as in Fig. 6, and presented as the mean (+SEM, N=4) fold change calculated by the average expression level of treated samples divided by that of time-matched controls. The relative significance of a LSD post hoc test after a significant one-way ANOVA between the stimulated and time-matched controls is shown above the bars as * $p \leq 0.05$, **$p \leq 0.01$ and *** $p \leq 0.001$.

Table 1. Primers used for 3'-RACE and real-time PCR analysis of gene expression

Gene	Primer name	Sequence (5' to 3')	Application
TNFR1a	TNFR1aF1	CGCGATTTAATACCCTGGCGTA	3'-RACE
	TNFR1aF2	GGTGACTCGACTGCATCGCC	3'-RACE
	TNFR1aF	CACATCCCACTCCCAATGGC	Real-time PCR
	TNFR1aR	CCTCTTGGATCAGCCTCCGAGT	Real-time PCR
TNFR1b	TNFR1bF1	ACTTAACACCCTGGCTTCTCTTGG	3'-RACE
	TNFR1bF2	TTTGGAGACTCGACTGCATCACT	3'-RACE
	TNFR1bF	GCACAGATCCCACTCCCAAACCT	Real-time PCR
	TNFR1bR	CCTCTTGGATCAGCCTCCGAGT	Real-time PCR
TNFR2a	TNFR2aF1	GACTTTTCTCAAGATCTTAAAGGGGCA	3'-RACE
	TNFR2aF2	CAAGATCTTAAAGGGGCAATAATAATTGT	3'-RACE
	TNFR2aF	GGAAATTGGGAGGCCATTAAATTTGATA	Real-time PCR
	TNFR2aR	GACTAGATGGCAAGGGACTGGTAGG	Real-time PCR
TNFR2b	TNFR2bF1	GCAAAGACGCCCTCAGTTTCC	3'-RACE
	TNFR2bF2	GGTTTCAGTATGGACTTTTTCGCAAT	3'-RACE
	TNFR2bF	GGAAAGGAGGCCATTAAATGTCTGC	Real-time PCR
	TNFR2bR	CAGATGTCAGAGGGCTGGTGGA	Real-time PCR
EF-1α	EF-1αF	CAAGGATATCCGTCGTGGCA	Real-time PCR
	EF-1αR	ACAGCGAAACGACCAAGAGG	Real-time PCR

Table 2. Summary of sequence features of rainbow trout TNFR1 and TNFR2 paralogues

Gene	TNFR1a	TNFR1b	TNFR2a	TNFR2b
cDNA sequence				
GenBank ID	HE717002	HE717003	HE717004	HE717005
Length (bp)	3027	2688	1814	2403
Upstream ATG	2	2	0	1
ATTTA motif	1	4	0	2
Genomic sequence				
Chromosome	Ch3	Ch3	Ch17	Ch7
GenBank ID	NC_035079	NC_035079	NC_035093	NC_035083
Length (bp)	12,367	9,901	24,309	7,461
Number of exons	10	10	9	9
Number of introns	9	9	8	8
Protein sequence				
Full length (aa)	403	397	455	463
Signal peptide	28	31	25	25
Mature peptide	375	366	430	438
TM region	217-241	215-239	249-272	281-304
N-glycosylation sites	2	1	3	2
Amino acid identity				
Trout TNFR1a	**100**	**80.2**	21.5	20.2
Salmon TNFR1a	**85.5**	**82.4**	21.9	21.2
Trout TNFR1b	**80.2**	**100**	21.5	19.4
Salmon TNFR1b	**79.8**	**93.0**	21.2	20.8
Pike TNFR1	**54.1**	**57.8**	22.2	21.2
Human TNFR1	**26.8**	**26.2**	22.0	21.9
Chicken TNFR1	**26.7**	**28.4**	20.0	19.3
Trout TNFR2a	21.7	21.5	**100**	**63.6**
Salmon TNFR2a	22.1	21.5	**91.2**	**64.4**
Trout TNFR2b	19.5	19.7	**63.6**	**100**
Salmon TNFR2b	21.7	21.3	**59.6**	**87.2**
Pike TNFR2	18.5	20.2	**46.2**	**45.9**
Human TNFR2	20.4	23.0	**28.6**	**30.1**
ChickenTNFR2	22.7	23.6	**29.9**	**27.6**

Figure 1


```
                  Signal peptide                                      CRD1
Trout  1a MDV--LRGKWKEKCILNVCALLILCWSVDP--SPLVPPLPTTQEFQCLEGSHYHT--ANGTCCRKCHEGFMLKEHCTKNGDNSLCVHCQEGRTYLEKSN
Salmon 1a MDV--LRGKWKEKCILNVCALLVLCWSVDP-----YLLAPPPEGSQCPDGSHYHN--ANGTCCRKCHEGFKLKEHCTKDGENSQCVPCEEGRTYREKSN
Trout  1b MDV--LRGKWKEKCILNVCALLVLCWSVDPSLS-APPPPDRTRELQCLEGSHYRT--ANGTCCRKCHEGFKLKEDCTKEGE-SQCEPCKEGRTYLERSN
Salmon 1b MDV--LRGKWKEKCILNVCTLLLLCWSVDLSLS-PPPPLDRTQELQCLEGSHYRN--ANGTCCRKCHEGFKLKKDCTKEGE-SQCEPCKEGKTYLERSN
Pike      MAV--LKGKWKEKNIFIVSTLLLMCWSVGLS---APPPSN-RTHSQCQEGSHYLS--KEGLCCGKCHQGFRLVKDCSVDNGNAECKPCSSG-TYRESSN
Catfish   MESRHHTGKWKKICAPCILFLLTVLRQCDA----TEGHKNFNNTGSCLEN-EYLH--GE-FCCDKCPPGFKLTRKCKGSGLRSECVKCSPG-SYQDNMN
Flounder  MEEAGHRGRRNKKAPVGT-TLLLMCMIVPTLAIS-----QPLVKPTCSPE-EYST--DKGICCNKCSPGYKLVEKCNAIGHRSKCAPCLPG-QFMDQLN
Medaka    MEGCGLQRTGNKKACVGI-FLLLMCTVL--YA------AQEPNQMKCPTG-DYLS--KNNICCNKCNPGYKLVKECLVAGHRSNCTSCPED-QYMDQIN
Human     MGLSTVPDLLLPLVLL---ELLVGIYPSGVIGLVPHLGDREKRDSVCPQG-KYIHPQNNSICCTKCHKGTYLYNDCPGPGQDTDCRECESG-SFTASEN
Mouse     MGLPTVPGLLLSLVLL---ALLMGIHPSGVTGLVPSLGDREKRDSLCPQG-KYVHSKNNSICCTKCHKGTYLVSDCPSPGRDTVCRECEKG-TFTASQN

             CRD2                                       CRD3                                    CRD4
Trout  1a YAKNCLRCTLC--VDNEEVFSPCKKSKNTVCRCKKGFYQNRINSGTRECLTCKTCGPGERETQPCKEESDTVCECKDFYFRDKKSNKTCLSCKLCELTA
Salmon 1a YVKTCLRCTLC--VDNEEEESPCKKSSNTLCRCKKGFYKNRINSETRECLSCKTCGPGERETQPCTQESDTVCECKDFYFRDKKNNKTCLPCQICELTA
Trout  1b YAKNCLRCTRCD-VDNEEEVSPCKKSRNTVCRCIEGFYKKRIDSVTRECLRCKTCGPGERETQPCTPESDTVCECKGF--RNKKNNRNCLPCQNCR-SA
Salmon 1b YAKNCLRCTRC--DDNEEEVSPCKKSRNTVCRCIEGFYKKRIDSVTRECLRCKTCGPGERETQPCTPERDTVCECMDF--RNQKNNRNCLPCQNCR-SA
Pike      AFRNCDSCRKC--VENEEVVSLCNRSRNQVCRCQTGFYLRKIDSETRECTSCETCGSGERVTQECTPESNTVCECSIFHYRDQMNKRTCLSCKNCT-ST
Catfish   HFQNCFSCRTCNPASNEIELAECTHMQDRKCGCKQGFYQEVLDDITMSCVPCRKCGVGEMETRSCNGQMNTECECKYNHYRVAK--RICAQCTNC--SS
Flounder  FYPNCKSCRICRASKHEHTLTKCVSKQNTICECDSGYYRFHIDSQAYECRKCAQCAPDEKEKQNCTPLKNTVCECKENYYRVK---NKCEPCKSC--TT
Medaka    YNPNCFRCKVCKRRKHEVEESSCKRDKNTVCVCEAGYYKSEIDPTAFECLKCSKCRPDEKVKQQCTRDTNTVCVCKDGHYREK---NTCKPCEGC--SV
Human     HLRHCLSCSKCRKEMGQVEISSCTVDRDTVCGCRKNQYRHYWSENLFQCFNCSLCLNGT-VHLSCQEKQNTVCTCHAGFFLRE---NECVSCSNCKKSL
Mouse     YLRQCLSCKTCRKEMSQVEISPCQADKDTVCGCKENQFQRYLSETHFQCVDCSPCFNGT-VTIPCKETQNTVCNCHAGFFLRE---SECVPCSHCKKNE

                                             Transmembrane domain
Trout  1a DCQQECS--SGTHPTPNGDGKSEDTGY----PYLLAAFSCVCLLLLVVVGIMGVLVVRRKRKGSNSFPSAEITSQVSETSTRRLIQ------------
Salmon 1a DCQQECS--SGTHPTPNGVAKPEDTGWDVWSPYLLALSVCVCVLLLVVVGIMGVLVVRRKPKGSSSFPSAEITSQGSETSTRRLIQ------------
Trout  1b DCQQECA--SGTDPTP----KPTDTGWGVWSPYLLAAFGCVCVVLLVVMGIMGVLVVRRKPKGSSSFLSAEITSQGSEKSTRRLIQ------------
Salmon 1b DCQQECA--SGTDPTP----KPEDTGWGVWSPYLLAAFGCVCVVLLVVMGIMGVLVVRRKPKGSNSFLSAEITSQGSEKSTRRLIQ------------
Pike      DCRQECHQ-ELATPST----VGVSVQPSNTSTTVLLLVFGCGFVFLLVVVMILACVVFWRV-KGLSSFPSDEVISQGT-KSTQKLIQ------------
Catfish   KCSDLCT--HGVKTTP----PSHPKSGYPLQTIVVLVWVCLCLVIGLPCIVSLYKGIKHWKK--RK---QNQYSQSS-ESHDPEKQ--GKETHKC----
Flounder  ECEHYCSGSSMNTKAP----DTGKE----FLTNIIAGVVSVALLLLGLVALITHLVTKRSIK--KKLVKPSHHDDSPDPCELILCS------------
Medaka    ECSHLCE--AVTTKRP----EPRDD----LMVAIVA-ISAVGVVMVALGVLVTHVFTKRFIK--KKMPSLTSQPTDISISERLFVH------------
Human     ECTKLCL----P-QIE-NVKGTEDSGTTVLLPLVI--FFGLCLLSLLFIGLMYRYQRWKSKL-YSIVCGKSTPEKEGELEGTTTKPLAPNPSFSPTPGF
Mouse     ECMKLCL----PPPLA-NVTNPQDSGTAVLLPLVI--LLGLCLLSFIFISLMCRYPRWRPEV-YSIICRDPVPVKE-EKAGKPLTP-APSPAFSPTSGF

                                                                                 Death domain
Trout  1a -------------------------------------------EDPENVLNQCIPSCSPVCECEQEPLRKLPDCVPKEIKISELIYSVLDQVPTRHMKE
Salmon 1a -------------------------------------------EDPENVLNQSIPSYSPVCESEQEPLSTLPDCVPKEIKISELIYSVLDQVPPRHVKE
Trout  1b -------------------------------------------EASENVLNQSIP----VCECEQELLSKLPDCVPKEIKISELIYSVLDQVPLRRVKE
Salmon 1b -------------------------------------------EDPENVLNQSIPSYSPVCESEQEPLRKLPACVPKEIKISELIYSVLDQVPLRRVKE
Pike      -------------------------------------------GQHGNVFIPTDLSSASVCE--RELLSKLPDCVPKEIKISDFIYSVLEQVPPRRVKE
Catfish   -------------------------------------------VQSKDDVSRLLPV---------QPDPVLPDCIPREIKTHEFVYLVLEIVPVSRFKE
Flounder  -----------------------------------------SEECSENSNVETTPN-SPVSE---QQPSNLPDCVPLEIKITDLIYSVLELVPALQVKQ
Medaka    -------------------------------------------EESSESQVESVPQ-SPVEE---QGQPNLPDCVPLEIRIPDLIYTVLDLVPVVRVKE
Human     TPTLGFS----PVPSSTFTSSSTYTPGDCPNFAAPRREVAPPYQGADPILATALAS-DPIPNPLQKWEDSAHKPQSLDTDDPATLYAVVENVPPLRWKE
Mouse     NPTLGFSTPGFSSPVSSTPISPIFGPSNW-HFMPPVSEVVPT-QGADPLLYESLCS-VPAPTSVQKWEDSAH-PQRPDNADLAILYAVVDGVPPARWKE

                             Death domain
Trout  1a LVRSLGVSDIVIERAENDHLRDTKEAQYQMLRVWAKGSAQ----GGGEVLARPLLYHLLDKLRDMDLGGTAEELETKYGDQ---------- 403
Salmon 1a LVRSLGVSDIVIERAENDHLRDTKEAQYQMLRVWAKGNAQ----GGGEVLARPLLYHLLDKLRDMDLGGTAEELETKYRDQ---------- 404
Trout  1b LVRSLGVSDIVIERAENDHLRDTKEAQYQMLRVWAKGSAQ----GGGEVLARPLLYHLLDKLRDMDLGGTAEELETKYGDQ---------- 397
Salmon 1b LVRSLGVSDIVIERAENDHLRDTKEAQYQMLRVWAKGNAQ----GGGGVLARPLLYHLLDKLRDMDLGGTAEELETKYGDQ---------- 400
Pike      LVRSLGVSDRVIELAENDYLRDTKEAQYQMLKFWACGGSQ----GRGGLLTLHLLHDLLVKLRNMELGGAAEELETIYGDYSGDK------- 400
Catfish   LVRRLNVSEQDIGRAERDN-RAFADAQYQMLMVWVDSGTR----GGKSILPHSLFQECVDRLKDMNLTACAESIEDKYA------------ 387
Flounder  LVRTLGVRDTEIEQAELDH-RFCKEAHYQMLRLWAEKVSRADGGGESGLLHLSLLQELLDKLRTMRLGGVAEELETKYSIQ---------- 393
Medaka    LVRSLGVRDTVIEQAEMDH-RQCREAQYQMLRVWAESGSHAAGGGRGDMLHLSLVKELLDKLRQMHLGGTAEELETKYGIH---------- 385
Human     FVRRLGLSDHEIDRLELQNGRCLREAQYSMLATWRRRTPR----------REATLELLGRVLRDMDLLGCLEDIEEALCGPAALPPAPSLLR 455
Mouse     FMRFMGLSEHEIERLEMQNGRCLREAQYSMLEAWRRRTPR----------HEDTLEVVGLVLSKMNLAGCLENILEALRNPAP-SSTTRLPR 454
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Figure 2

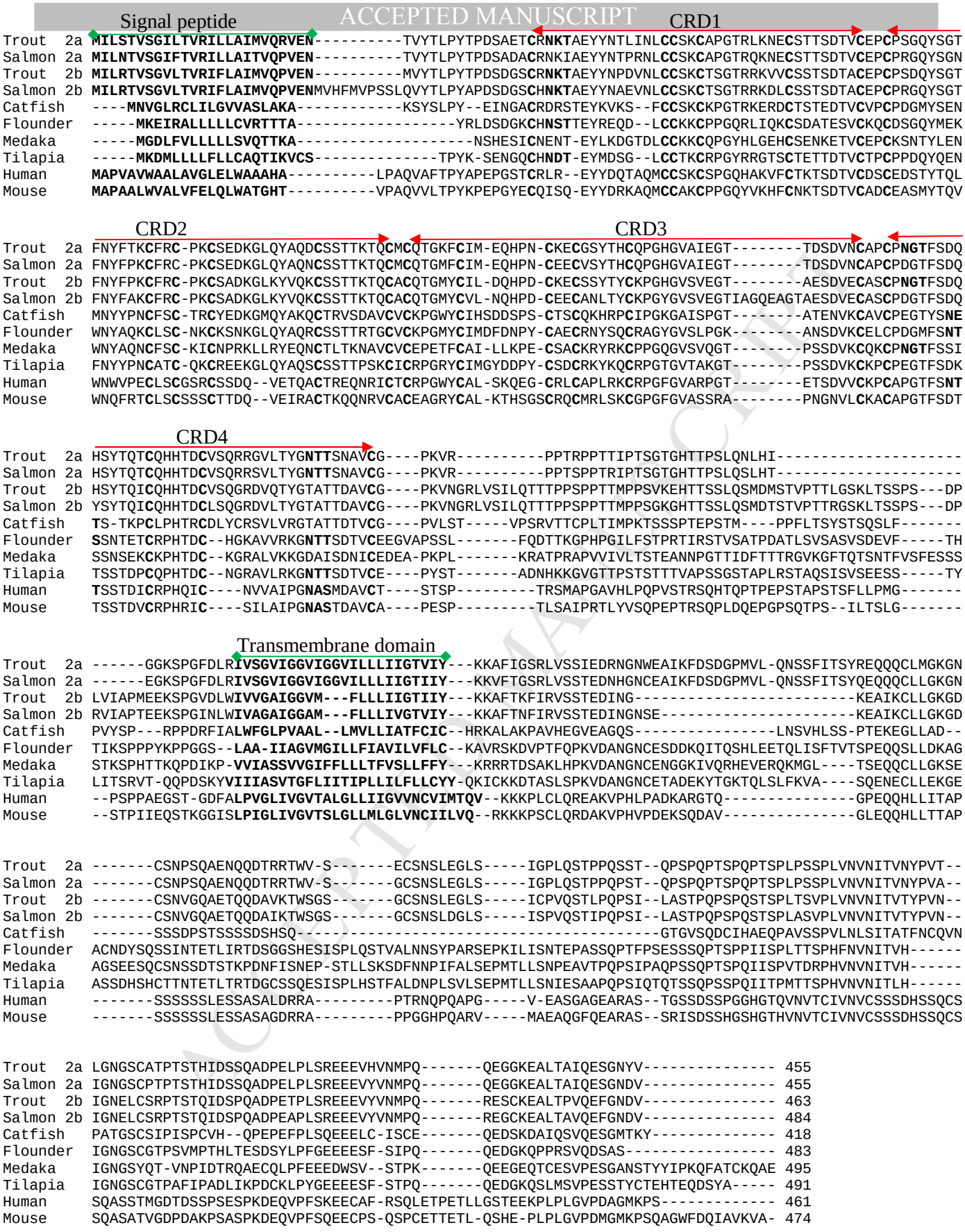
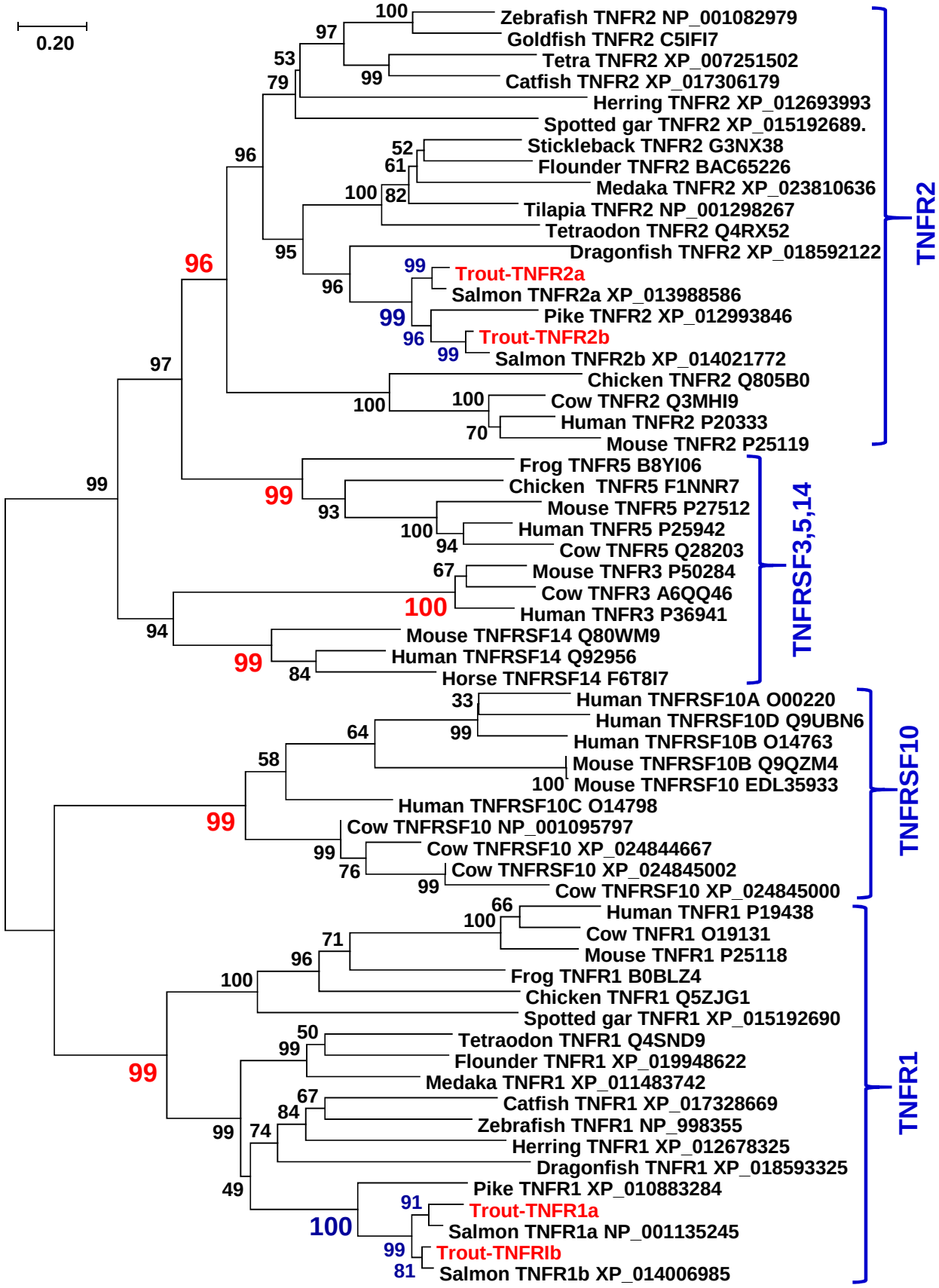


Figure 3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Goldfish TNFR2 C5IFI7
Tetra TNFR2 XP_007251502
Catfish TNFR2 XP_017306179
Herring TNFR2 XP_012693993
Spotted gar TNFR2 XP_015192689.
Stickleback TNFR2 G3NX38
Flounder TNFR2 BAC65226
Medaka TNFR2 XP_023810636
Tilapia TNFR2 NP_001298267
Tetraodon TNFR2 Q4RX52
Dragonfish TNFR2 XP_018592122
Trout-TNFR2a
Salmon TNFR2a XP_013988586
Pike TNFR2 XP_012993846
Trout-TNFR2b
Salmon TNFR2b XP_014021772
Chicken TNFR2 Q805B0
Cow TNFR2 Q3MHI9
Human TNFR2 P20333
Mouse TNFR2 P25119
TNFR2
Frog TNFR5 B8YI06
Chicken TNFR5 F1NNR7
Mouse TNFR5 P27512
Human TNFR5 P25942
Cow TNFR5 Q28203
Mouse TNFR3 P50284
Cow TNFR3 A6QQ46
Human TNFR3 P36941
Mouse TNFRSF14 Q80WM9
Human TNFRSF14 Q92956
Horse TNFRSF14 F6T8I7
TNFRSF3,5,14
Human TNFRSF10A O00220
Human TNFRSF10D Q9UBN6
Human TNFRSF10B O14763
Mouse TNFRSF10B Q9QZM4
Mouse TNFRSF10 EDL35933
Human TNFRSF10C O14798
Cow TNFRSF10 NP_001095797
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Human TNFR1 P19438
Cow TNFR1 O19131
Mouse TNFR1 P25118
Frog TNFR1 B0BLZ4
Chicken TNFR1 Q5ZJG1
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Tetraodon TNFR1 Q4SND9
Flounder TNFR1 XP_019948622
Medaka TNFR1 XP_011483742
Catfish TNFR1 XP_017328669
Zebrafish TNFR1 NP_998355
Herring TNFR1 XP_012678325
Dragonfish TNFR1 XP_018593325
Pike TNFR1 XP_010883284
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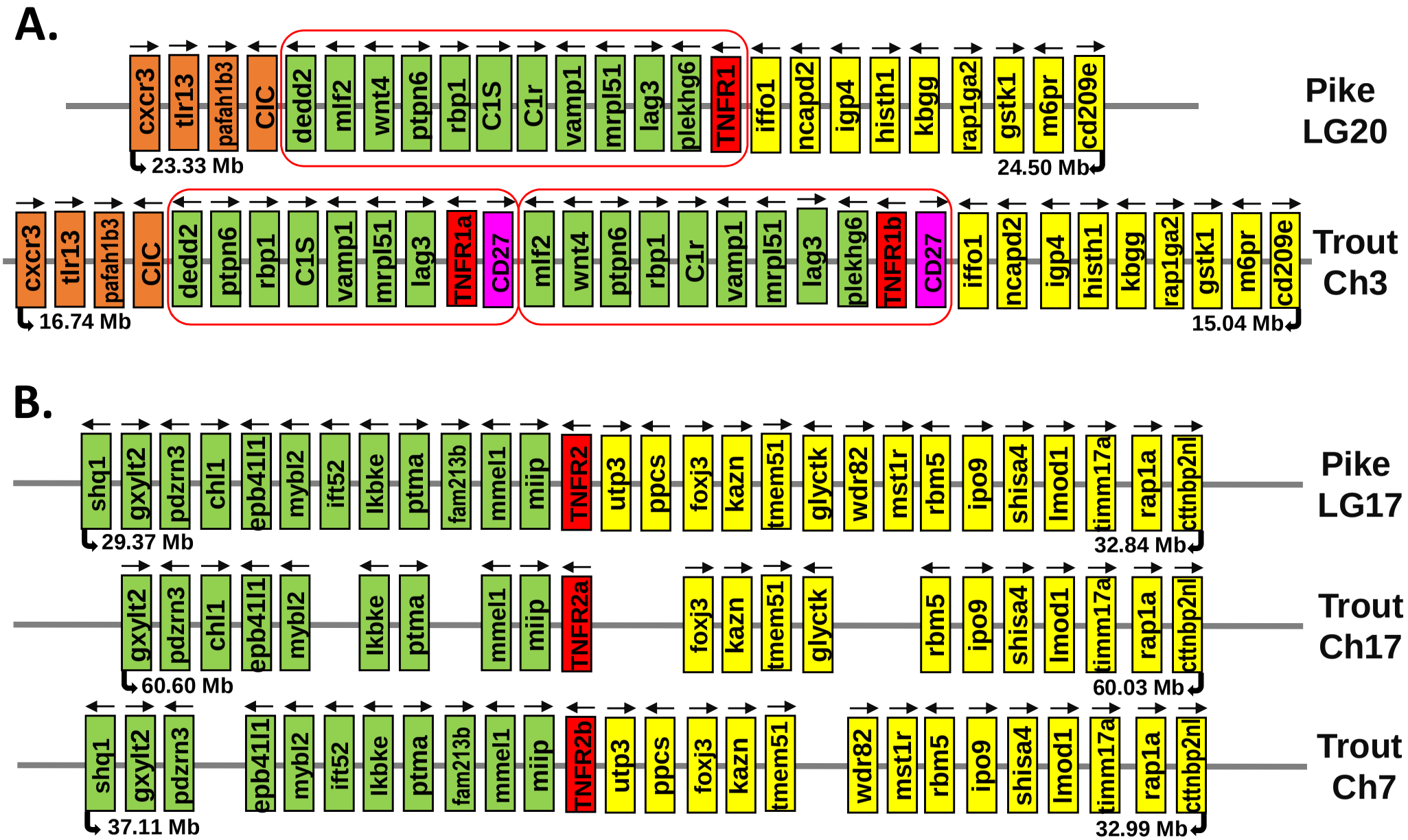
Salmon TNFR1a NP_001135245
Trout-TNFRIb
Salmon TNFR1b XP_014006985
TNFR1

Figure 4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wnt4
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C1r
vamp1
mrpl51
lag3
plekhg6
TNFR1
iffo1
ncapd2
igp4
histh1
kbgg
rap1ga2
gstk1
m6pr
cd209e
23.33 Mb
24.50 Mb
Pike LG20
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tlr13
pafah1b3
CIC
dedd2
ptpn6
rbp1
C1S
vamp1
mrpl51
lag3
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CD27
mlf2
wnt4
ptpn6
rbp1
C1r
vamp1
mrpl51
lag3
plekhg6
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CD27
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ncapd2
igp4
histh1
kbgg
rap1ga2
gstk1
m6pr
cd209e
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Trout Ch3
B.
shq1
gxylt2
pdzrn3
chl1
epb41l1
mybl2
ift52
lkbke
ptma
fam213b
mmel1
miip
TNFR2
utp3
ppcs
foxj3
kazn
tmem51
glyctk
wdr82
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lmod1
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Pike LG17
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pdzrn3
chl1
epb41l1
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ptma
mmel1
miip
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ptma
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miip
TNFR2b
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ppcs
foxj3
kazn
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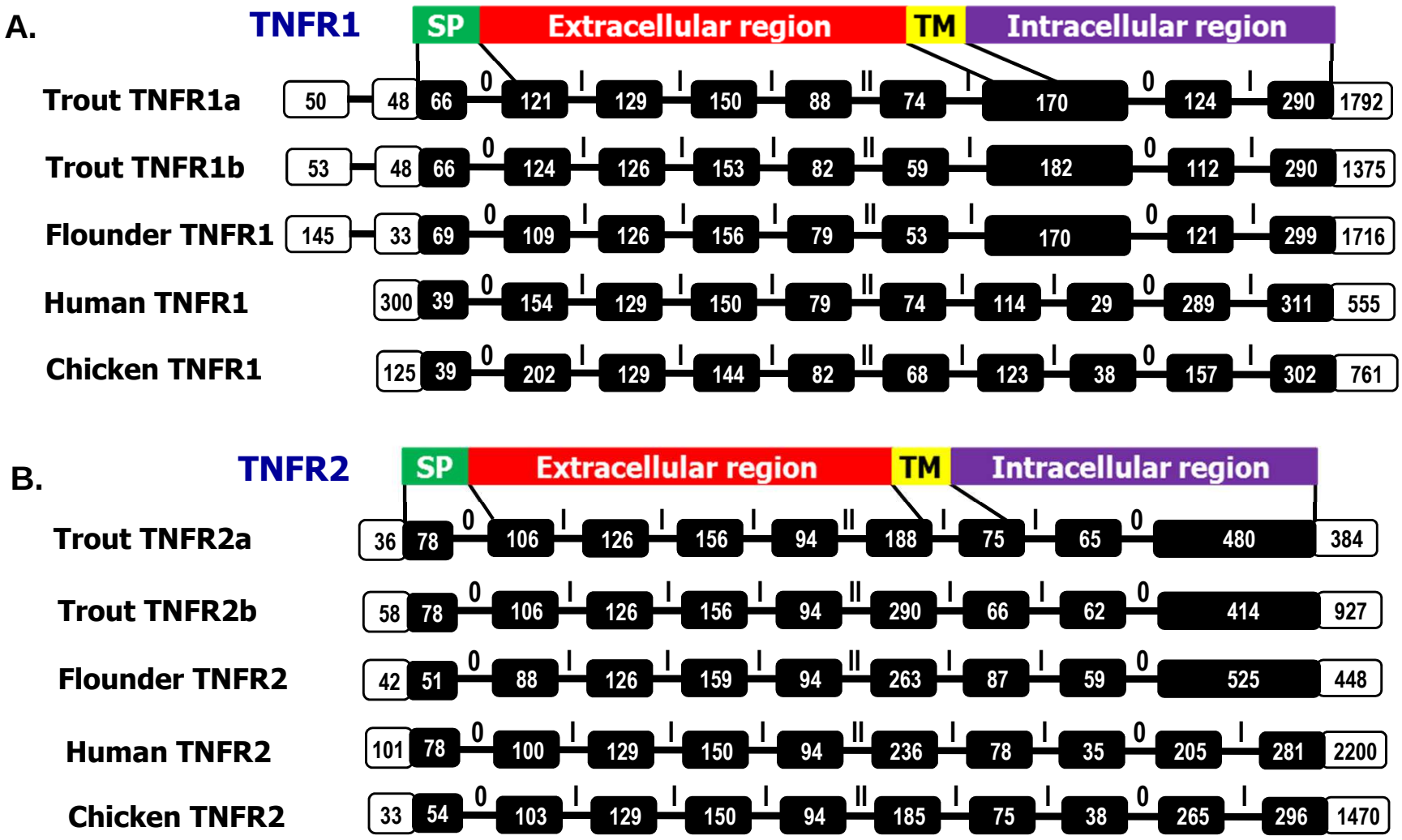
32.99 Mb
Trout Ch7

Figure 5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TNFR1
SP
Extracellular region
TM
Intracellular region
Trout TNFR1a
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Trout TNFR1b
53 48 66 0 124 I 126 I 153 I 82 II 59 I 182 0 112 I 290 1375
Flounder TNFR1
145 33 69 0 109 I 126 I 156 I 79 II 53 I 170 0 121 I 299 1716
Human TNFR1
300 39 0 154 I 129 I 150 I 79 II 74 I 114 I 29 0 289 I 311 555
Chicken TNFR1
125 39 0 202 I 129 I 144 I 82 II 68 I 123 I 38 0 157 I 302 761
B.
TNFR2
SP
Extracellular region
TM
Intracellular region
Trout TNFR2a
36 78 0 106 I 126 I 156 I 94 II 188 I 75 I 65 0 480 384
Trout TNFR2b
58 78 0 106 I 126 I 156 I 94 II 290 I 66 I 62 0 414 927
Flounder TNFR2
42 51 0 88 I 126 I 159 I 94 II 263 I 87 I 59 0 525 448
Human TNFR2
101 78 0 100 I 129 I 150 I 94 II 236 I 78 I 35 0 205 I 281 2200
Chicken TNFR2
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Figure 6

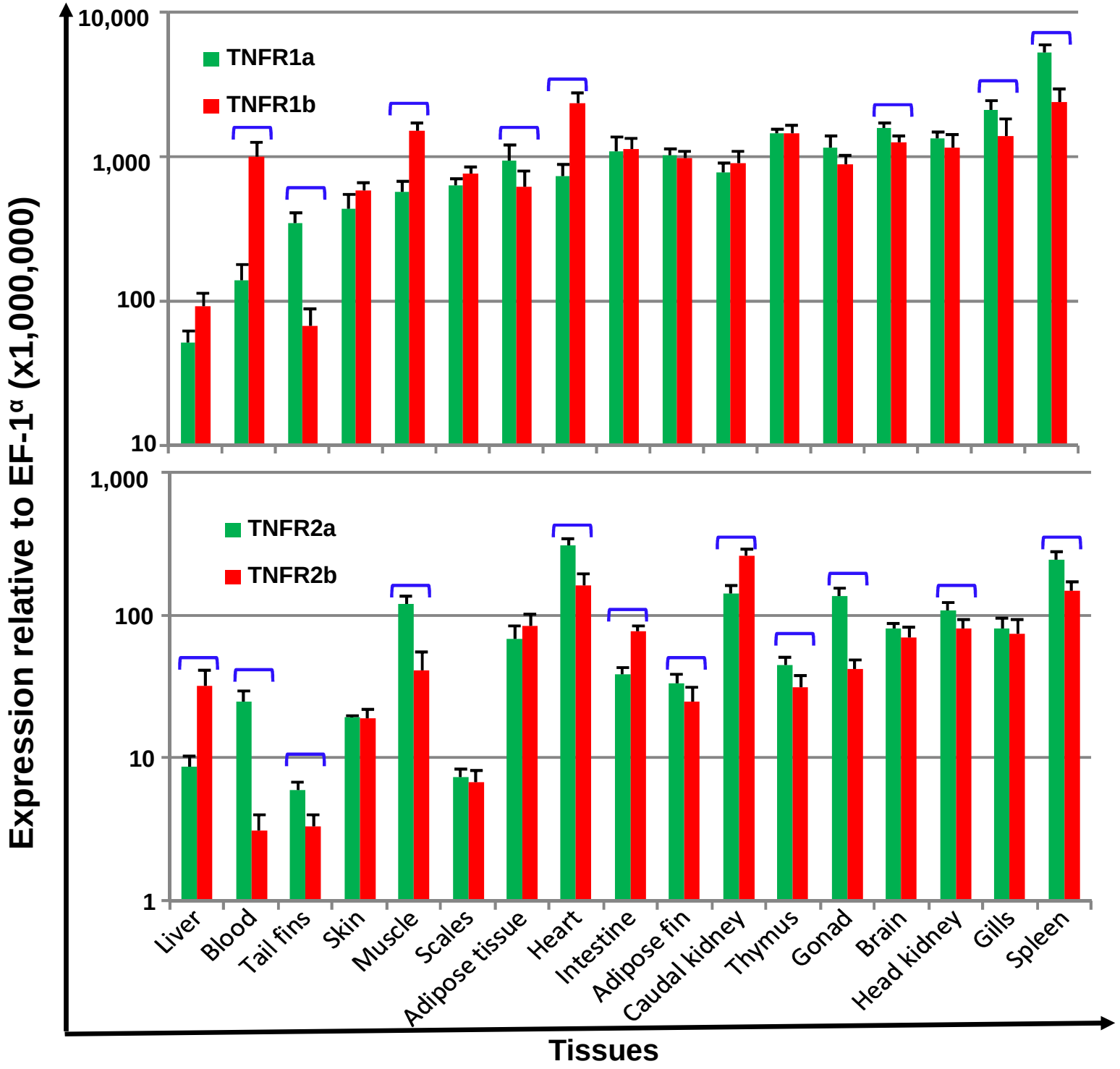
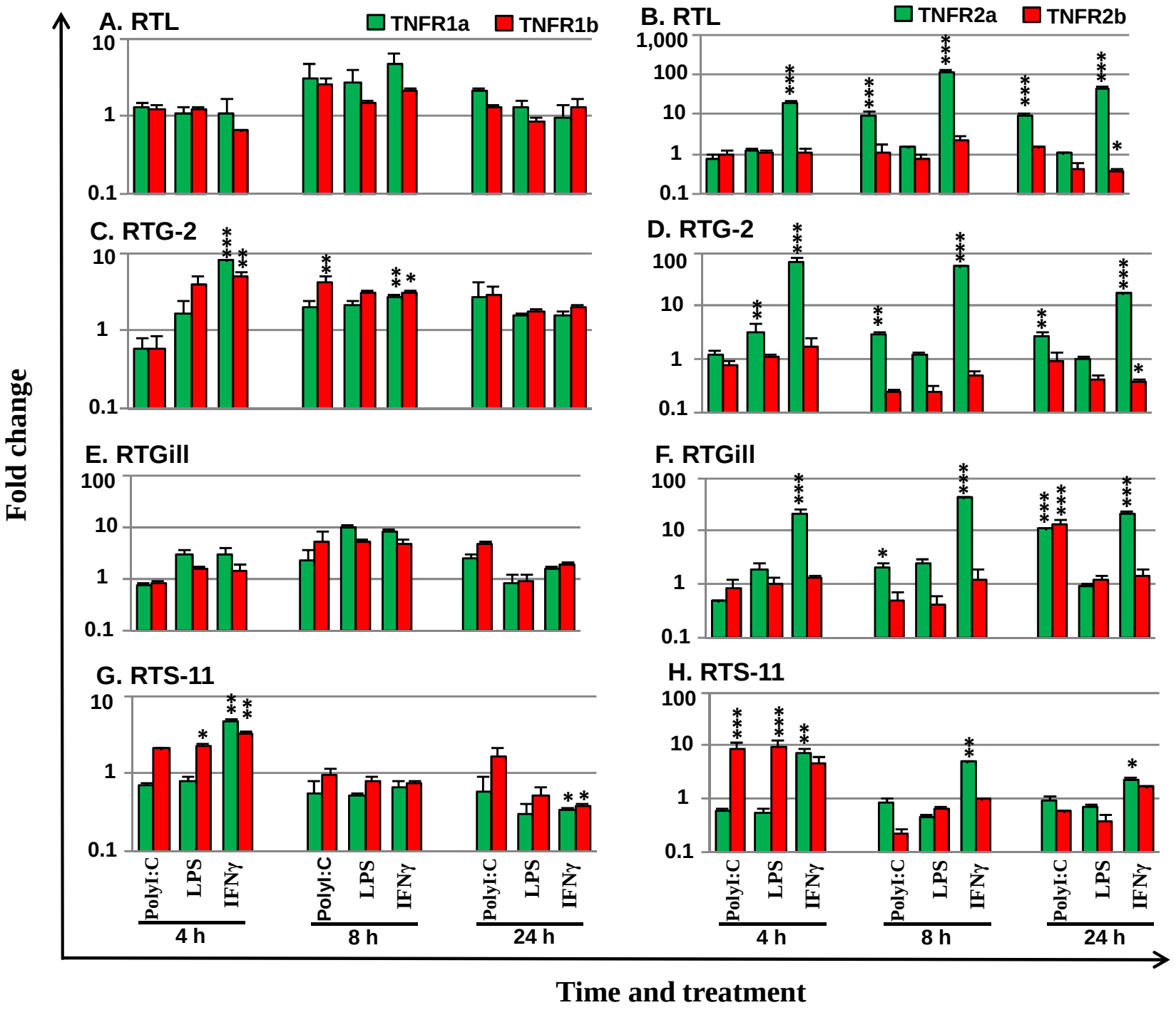

Expression relative to EF-1α (x1,000,000)
10,000
1,000
100
10
TNFR1a
TNFR1b
1,000
100
10
1
TNFR2a
TNFR2b
Liver
Blood
Tail fins
Skin
Muscle
Scales
Adipose tissue
Heart
Intestine
Adipose fin
Caudal kidney
Thymus
Gonad
Brain
Head kidney
Gills
Spleen
Tissues

Figure 7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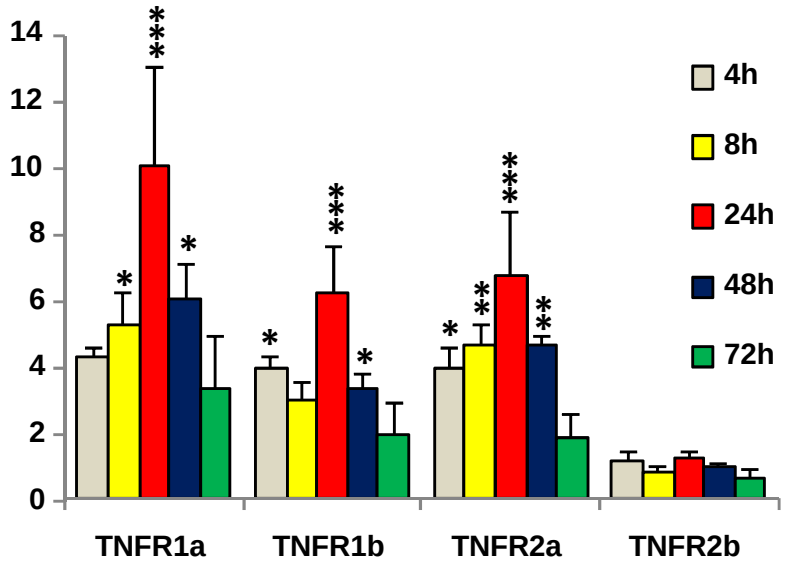
TNFR1a
TNFR1b
B. RTL
TNFR2a
TNFR2b
C. RTG-2
D. RTG-2
E. RTGill
F. RTGill
G. RTS-11
H. RTS-11
Fold change
PolyI:C
LPS
IFNγ
4 h
8 h
24 h
Time and treatment

Figure 8



Highlights

1. Two paralogues for each of TNFR1 and TNFR2 are present in salmonids.
2. TNFR2 paralogues arose from the 4R WGD but TNFR1 paralogues arose from an en bloc duplication.
3. Teleost and tetrapod TNFR1 and TNFR2 orthologues differ in exon/intron structure.
4. Trout TNF receptors are ubiquitously expressed with high level expression in immune organs.
5. Trout TNF receptors can be differentially modulated by PAMPs and cytokines.