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ST2 from rainbow trout quenches TLR signalling, localises at the nuclear membrane and allows the nuclear translocation of MYD88

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ABSTRACT

The mammalian interleukin 1 receptor-like 1 receptor (IL1RL1), commonly known as ST2, is thought to downregulate TLR signalling by sequestering the signalling adapter MYD88 (myeloid differentiation primary response protein 88). ST2 sequences are known in several fish species, but none of them have functionally been examined. We characterised ST2 from rainbow trout (*Oncorhynchus mykiss*) and the structure of its encoding gene. The primary sequence of ST2 is only weakly conserved from fish to human. However, the amino acid sequences forming the interfaces for ST2 and MYD88 interaction are well conserved throughout evolution. High similarity of the gene segmentation unambiguously proves the common ancestry of fish and mammalian ST2. Trout *ST2* and trout *MYD88* genes were constitutively expressed in embryonic, larval and adult trout. In vivo infection with *Aeromonas salmonicida* did not modulate the mRNA levels of both factors. Overexpressing trout ST2 in the mammalian HEK-293 reconstitution system of TLR2 signalling quenched the *Escherichia coli*–induced activation of NF-κB and SAA promoters in a dose-dependent fashion. The expression of GFP-tagged trout ST2 in human HEK-293 or trout CHSE-214 cells surprisingly revealed that (i) ST2 localised abundantly at the nuclear membrane rather than at the cell membrane and (ii) the coexpression of both ST2 and MYD88 allowed the translocation of trout MYD88 from cytoplasm to nucleus, as assessed using confocal microscopy and Western blotting. Hence, we validated that trout ST2 is a dampener of TLR signalling and interacts with MYD88. The spatial distribution of these factors raises questions about how this repressive mechanism functions.

1. Introduction

ST2 is encoded by the interleukin 1 receptor-like 1 gene (*IL1RL1;* HUGO gene ID: **5998**). It was originally described as serum stimulation 2 factor revealing induced expression after stimulating the proliferation of quiescent murine BALB/c-3T3 cells (Tominaga, 1989). Recent reviews (Lott et al., 2015; Oboki et al., 2010) comprehensively document that the mammalian ST2 factor and its variants exert multiple functions by contributing to cell proliferation (Tominaga et al., 2015); constituting part of the receptor for interleukin IL33 (Schmitz et al., 2005) thereby transmitting its functions during inflammation and allergy; and dampening pathogen-induced signalling from toll-like receptors (TLRs; Brint et al., 2004).

Several protein variants (ST2L, sST2, and ST2V) of the mammalian factor are expressed in a cell-type-specific fashion, depending on the activity of either a distal or a proximal promoter (Bergers et al., 1994; Iwahana et al., 1999; Lipsky et al., 2012). The longest protein variant ST2L (Yanagisawa et al., 1993) represents a transmembrane receptor with significant similarity to the interleukin-1 receptor (IL1R). On its extra-cellular part, it features three immunoglobulin (Ig)–like domains for ligand binding, a transmembrane (TM) domain and on the cytoplasmic part of the receptor a characteristic toll-like/interleukin-1 receptor resistance (TIR) domain involved in factor interaction. In mammals, ST2L is predominantly expressed by hematopoietic cell lineages, notably in T_H2 and mast cells. Membrane-bound ST2L may heterodimerise with the co-receptor IL1RAP (IL1R accessory protein) to constitute the receptor for IL33 on T_H2- and mast cells (Schmitz et al., 2005). The activated ternary signalling complex ST2L-IL1RAP-IL33 recruits TIR domain-containing adapters, which in turn activate MAPK

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Abbreviations

aa	amino acids
CHSE	Chinook salmon embryo
CMV	cytomegalovirus
cds	coding sequence
DIC	differential interference contrast
ELAM	endothelial leukocyte adhesion molecule 1
GFP	green fluorescent protein
HEK	human embryonic kidney
Ig	immunoglobulin
IL	interleukin
IL1R	interleukin-1 receptor
IL1RL1	interleukin-1 receptor-like 1
LPS	lipopolysaccharide
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NLS	nuclear localisation signals
ORF	open reading frame
RT-qPCR	real-time quantitative reverse transcriptase polymerase chain reaction
SAA	serum amyloid A
T_H2	T helper cell type 2
TIR	toll/interleukin-1 receptor resistance domain
TM	transmembrane
TLR	toll-like receptor

kinases (Milovanovic et al., 2012; Tare et al., 2010) to mediate the T_H2-polarising function of IL33.

The 'soluble' form sST2 (Tominaga et al., 1989) features only the N-terminal extracellular Ig-like domains of the receptor lacking both, the TM and the TIR domain. It is expressed by both hematopoietic and non-hematopoietic cells and functions as a decoy receptor for IL33 (Hayakawa et al., 2007) quenching IL33 functioning. Two more variants, ST2V (Tominaga et al., 1999) lacking the third immunoglobulin-like domain and the bird-specific ST2LV (Iwahana et al., 2004) lacking the transmembrane region have been described, but their physiological function is still unknown.

Mammalian ST2 has been demonstrated to dampen the overshooting responses of IL1R, toll-like receptor 2 (TLR2), TLR4, and TLR9 signalling (Brint et al., 2004, 2002; Liu et al., 2010) by binding and sequestering MYD88. Individual members of the large TLR family of pathogen receptors (Akira et al., 2006; Rebl et al., 2010; Roach et al., 2005) sense and bind distinct pathogen-associated molecular patterns (PAMPs) of invading microbes (De Nardo, 2015; O'Neill, 2008). Subsequently, TLRs recruit to their TIR domain MYD88 and other factors into a post-receptor signalling complex known as Myddosome (De Nardo, 2015; Lin et al., 2010). This establishes an indispensable structural platform for transmitting the pathogen signal into the cytoplasm to eventually activate the NF-κB complex of transcription factors. These are known as master switch for immune gene regulation (Hayden and Ghosh, 2011; Karin and Lin, 2002). Protein docking studies proved that ST2L physically binds to MYD88 *via* the interaction of their TIR domains (Brint et al., 2004) thereby masking the TLR-MYD88 interacting sites (Basith et al., 2011).

Little is known about the structural variants and function of ST2 from fish. ST2 sequences from several fish species have been deposited in the databases and only a single annotation report revealed some structural similarities of the salmon ST2 receptor to that of higher vertebrates (Stansberg et al., 2003). However, the conservation of ST2 throughout evolution appeared to be low. Particularly poor conservation was obvious for the N-terminal (IL33 binding) domain of the factor, possibly reflecting that teleosts do not encode IL33 (Ogryzko et al., 2014).

Extending our previous characterisations of negative regulators of TLR signalling in rainbow trout *Oncorhynchus mykiss* (Rebl et al., 2011a, 2011b, 2008), we were interested to learn whether ST2 from trout would also down-regulate TLR signalling, possibly involving sequestration of MYD88. We found that trout and mammalian ST2-encoding genes are true orthologs, and the factors share the function to dampen TLR signalling. However, our surprising observation that the trout ortholog localises at the nuclear membrane rather than at the cell membrane offers no clue to understand at the molecular level how that dampening mechanism of TLR signalling operates.

2. Materials and methods

2.1. Fish, sampling and nucleic acid extraction

Rainbow trout (strain BORN, Germany) were kept at the Institute for Fisheries LFA-MV (Born and Hohen Wangelin, Germany). Eggs were kept at 8 °C in upwelling incubators and sampled at 21 days postfertilisation (dpf) as 'eyed eggs'. Subsequently, larvae with yolk sac were maintained at 14 °C in standard troughs and collected at 42-dpf and 49-dpf. Fry fish without yolk sacs were sampled at 56-dpf; adult rainbow trout were maintained at 16°C–18 °C in 300-L tanks and sampled at 11 months of age. At that time, the fish had an average length of 27.4 ± 1.1 cm and a weight of 309.3 ± 38.8 g. Eggs, larvae and tissue samples from adult fish (brain, gills, head kidney, liver, muscle, spleen and trunk kidney) were transferred into RNAlater (Qiagen, Hilden, Germany) and subsequently stored at −80 °C.

The peritoneal injection of rainbow trout with 1×10^7 *Aeromonas salmonicida* ssp. *salmonicida* (wild-type strain JF 2267) has already been described by Brietzke et al. (2014). Samples were taken from four individuals at 0, 6, 12 and 72 h postinfection and immediately snap-frozen in liquid nitrogen.

RNA was extracted (TRIzol, Invitrogen) from samples powdered under liquid N_2 in a mortar and purified with the RNeasy Mini Kit (Qiagen) including the in-column DNase treatment for 30 min. DNA was isolated from kidney samples using the DNeasy Blood and Tissue Kit (Qiagen). RNA and DNA purity and concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The integrity of RNA was validated in denaturing agarose gels.

2.2. Isolation of ST2-and MYD88-encoding sequences from rainbow trout

All oligonucleotides (listed in Suppl. Table 1) were designed with the PSQ Assay Design software (Biotage) and were purchased from Sigma-Aldrich. The salmon *ST2* (*IL1RL1*) cDNA sequence (**NM_001123550**) served to derive oligonucleotides for the amplification of trout ST2-specific cDNA and gene fragments. Total RNA from liver, spleen and gills was pooled and then reverse-transcribed with a GeneRace Kit (Life Technologies) according to the manufacturer's instructions. Briefly, RNA was dephosphorylated, decapped and finally ligated to an adapter oligonucleotide. Reverse transcription was carried out using the SuperScript II Reverse Transcriptase (Life Technologies). The subsequent RACE experiments were performed using the specific primers OM_ST2_3end_f1/f2 and OM_ST2_5end_r, respectively (Suppl. Table 1).

PCR products were generated with the HotStarTaq Plus DNA Polymerase (Qiagen) following standard protocols and subsequently

Table 1
Comparison of trout ST2 protein sequence (599 aa) with putative orthologs from selected vertebrate species.

Species	Length [aa residues]	Sequence similarity [%]			
		Total	Ig/Ig-like	TM	TIR
			68–365[a]	368–390[a]	412–589[a]
Salmo salar	591	93.9	95.3	90.9	93.8
Esox lucius	540	67.2	64.8	63.2	75.0
Oreochromis nilocticus	565	42.0	36.6	45.0	58.3
Danio rerio	561	40.1	36.9	54.5	50.6
Gallus gallus	533	29.4	26.7	35.7	33.1
Homo sapiens	556	27.6	22.9	23.8	37.9
Mus musculus	567	25.9	22.2	38.9	33.9

[a] Indicates aa position in the trout factor.

purified using the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions. The cDNA amplificates were all cloned into pGEM-T Easy vector (Promega) and sequenced (MegaBACE; GE Healthcare).

Segments of the *ST2* gene were amplified from genomic DNA in successive steps, applying a collection of gene-specific oligonucleotides, which were all derived from our *ST2* cDNA sequences. Intron sequences were eventually retrieved with PCR techniques using as template a genomic walking library from rainbow trout DNA prepared with the GenomeWalker Universal Kit (Takara Bio USA) and suitable trout-specific oligonucleotide primers (Suppl. Table 1). The resulting fragments were cloned and sequenced.

Each nucleotide position of both cDNA and genomic DNA fragments was sequenced at least three times using different cDNA and genomic DNA preparations.

2.3. Structural characterisation of the ST2-encoding gene

Exon-intron boundaries were revealed by comparing the ST2-encoding cDNA and genomic sequences. Introns in orthologous genes were retrieved by consulting the Ensembl database (http://www.ensembl.org). The cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) identified nuclear localisation signals (NLS) within the trout MYD88 sequence with a score of 5.2. Local protein sequence alignments were performed using the EMBOSS matcher algorithm version 2.0u4. Functional motifs and domains of conceptually translated amino acid (aa) sequences (Expert Protein Analysis System proteomics server, Swiss Institute of Bioinformatics; http://www.expasy.ch) were predicted using the SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de) and NCBI (National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov) protein databases. The putative transmembrane domain of trout ST2 was searched for by using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis package (MEGA 5; Tamura et al., 2011). The dendrogram was reconstructed using the neighbour-joining method based on Poisson-corrected distances. Node robustness was evaluated on a bootstrap analysis based on 1000 iterations.

2.4. Quantitative PCR

For real-time fluorescence-based quantitative PCR (RT-qPCR), two gene-specific primer pairs were designed (Suppl. Table 1), one of which was spanning an exon-intron boundary (SS_ST2_LC_f, OM_MYD88_LC_r). SuperScript II Reverse Transcriptase (Life Technologies) was used to generate the cDNA from 1.5 μg of total RNA. The cDNA was column-purified (High Pure PCR Product Purification Kit, Roche) and aliquoted such that each assay received a cDNA input as derived from 150 ng of total RNA. Quantification was performed on the LightCycler 480 System (Roche) using the SensiFAST SYBR No-ROX Kit (Bioline). Correctness of amplificates was validated by cloning and sequencing. The quality of the products was validated after each LightCycler run by resolving the samples on agarose gels. Relative copy numbers were titrated against external standards consisting of a dilution series (10^7–10^2 copies) of the cloned amplicons.

Levels of transcripts encoding *EEF1A1* (eukaryotic translation elongation factor 1 alpha 1; Bowers et al., 2008) and *RPS5* (ribosomal protein S5; Suppl. Table 1) did not vary among the different tissue samples from adult fish and were therefore used for normalisation. In contrast, cDNA copy numbers from embryos and larvae were only normalised against the input of total RNA concentration into the reverse transcription (Bustin, 2000) since neither of the commonly used housekeeping reference genes *EEF1A*, *RPS5*, *ACTB*, *GAPDH*, *RPL4*, *RPS20* or *UBA52* showed consistent expression levels between different developmental stages.

2.5. Construction of ST2 and MYD88 expression plasmids

We established clones expressing fluorescence-tagged ST2 and MYD88 factors from rainbow trout to analyse their function and spatial arrangement in cellular substructures. The entire ORF encoding trout ST2 was cloned *via Xho*I and *Eco*RI into the vector pAsRed2-N1 (Takara Bio USA) using the oligonucleotides OM_ST2_XhoI_f and OM_ST2_Eco_r (Suppl. Table 1) and standard PCR procedures using the Platinum Taq High-Fidelity DNA-Polymerase (Thermo Fisher Scientific). Thus, the red fluorescent protein was C-terminally fused to the full-length ST2 factor.

We generated ST2 expression clones without fused AsRed2 protein since the AsRed2 tag might modulate the efficacy of the ST2 function. Therefore, a translation stop codon was inserted behind the ST2 ORF in the ST2-AsRed2 expressing vector. We inserted a double-stranded (ds) oligonucleotide harbouring in frame a translation stop codon into the 5′-*Not*I 3′-*Eco*RI sites residing between the ST2 and the AsRed2 encoding sequences. This ds-oligonucleotide was formed by annealing the complementary oligonucleotides AATTCTA**TGA**GC and GGCCGC**TCA**TAG and featured *Eco*RI and *Not*I overhangs (underlined). It was ligated into the appropriately digested hosting vector.

The trout MYD88 factor was tagged with the green fluorescence protein by amplifying the entire MYD88 ORF (886 bp) with the primers OM_MYD88_Spe_f and OM_MYD88_Eco_r (Suppl. Table 1) and finally cloned into those restriction sites of the vector pAM505 GFP (AF140578; Takara Bio USA), fusing MYD88 N-terminally in front of the GFP protein.

All plasmid DNA was prepared endotoxin-free using the Endo-Free Plasmid Maxi Kit (Qiagen), and the correctness of all constructs was validated through sequencing.

2.6. Monitoring the efficacy of TLR signalling in HEK-293 cells

TLR signalling ultimately activates NF-κB factors (Kawai and Akira, 2007). We therefore used the reconstitution of TLR2 signalling in the human embryonic kidney cell line 293 (HEK-293; ATCC) to evaluate the impact of ST2 and MYD88 on the strength of TLR signalling. Maintenance of HEK-293 cells in EMEM, their transfection (Lipofectamine 2000; Invitrogen) with a vector expressing the bovine TLR2 factor, the NF-κB-driven and Renilla luciferase–expressing ELAM reporter gene (recloned from pNiFty-SEAP [InvivoGen]), and challenging the transfected cells with heat-killed particles of the mastitis causing *E. coli* strain 1303 were

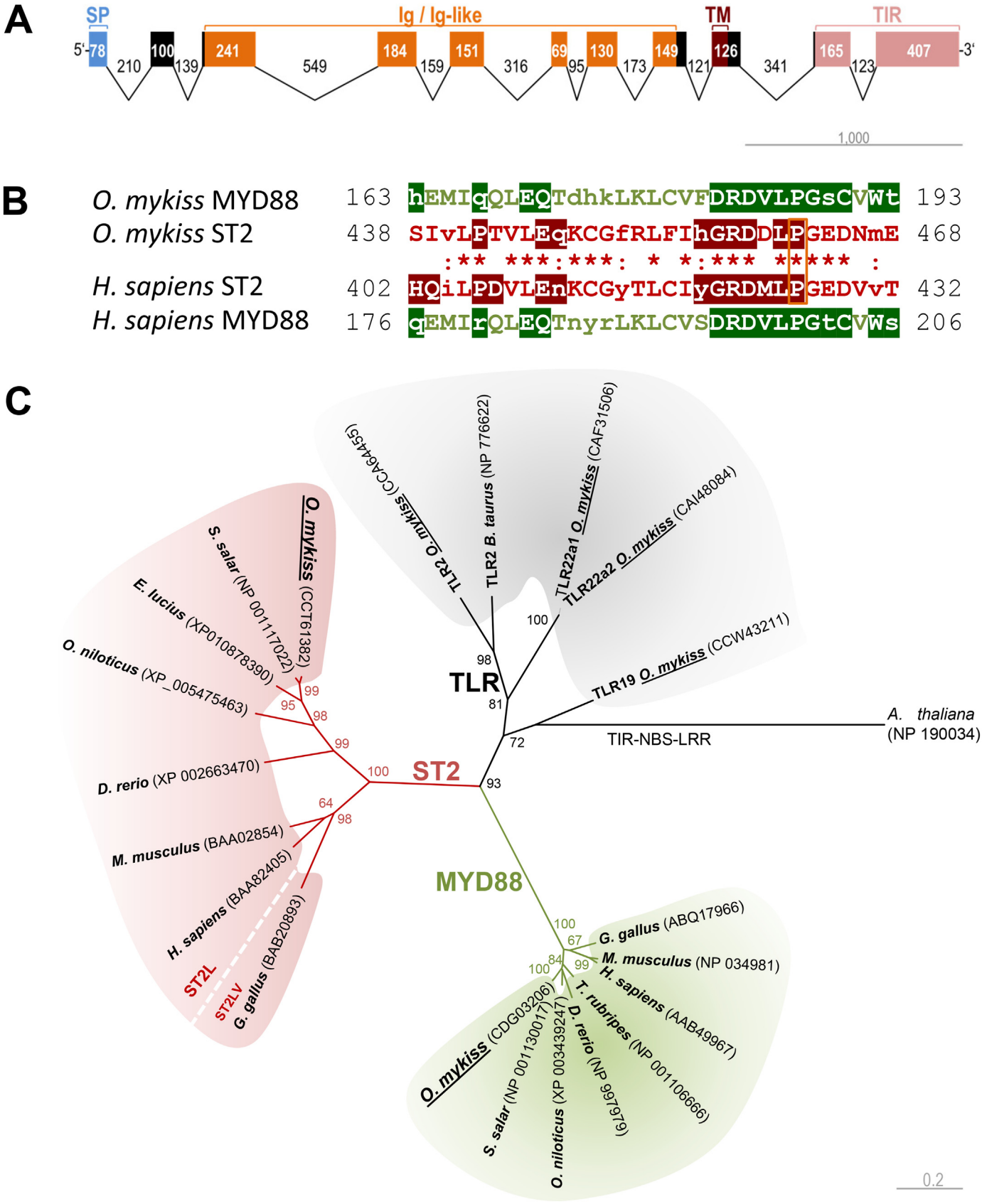

Fig. 1. (A) Genomic organisation of the trout gene encoding ST2 (IL1RL1). Exons are represented by filled boxes. Encoded domains are labelled above the scheme (blue, SP, signal peptide; red, Ig/Ig-like; dark red, TM, transmembrane; pink, TIR, TIR domain). Lines between exons represent introns. Exon and intron sizes are drawn to scale and given in base pairs [bp]. The depicted area covers the cds only; the scale bar indicates 1000 bp. **(B) Alignment of that section of human and trout MYD88 and ST2 factors known to forming the physical contact sites between both factors** (Basith et al., 2011). Potentially interacting residues of trout ST2 and MYD88 factors are highlighted with a red and green underlay,

previously described (Brietzke et al., 2014; Yang et al., 2008). We used in some experiments a firefly luciferase–expressing reporter gene to monitor the activity of the trout SAA promoter (Rebl et al., 2011b). The amount of transfected DNA was kept constant in all titration assays by eventually filling up with enough DNA of the empty cloning vector pcDNA3.1 (+).

The luciferase activity of the reporter genes was assessed using the Dual-Luciferase Reporter Assay System (Madison) and a luminometer instrument (Lumat^3 LB 9508 Single Tube Luminometer, Berthold). The results were normalised against the protein content of cell extracts as determined with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The spectrophotometer readings had previously been calibrated against the Bio-Rad protein detection assay (Bio-Rad Laboratories). Each quantitative expression experiment was assayed in triplicate and replicated three times.

HEK-293 cells stably expressing either the GFP-tagged MYD88 or the AsRed2-tagged ST2 factor were established in 9 cm dishes by cotransfecting 4 μg either of the expressing vectors each together with 400 ng of the pSV2neo vector conferring resistance to G418 (InvivoGen). Transfected cultures were grown in selection medium (EMEM supplemented with 200 μg/ml G418) for more than 2 weeks. After this selection period, microscopic examination revealed that more than 80% of the cells expressed the respective factor. Pools containing >500 individual clones were stored frozen in liquid N_2.

Embryonic CHSE-214 cells of the Chinook salmon (*Oncorhynchus tshawytscha*). This species belongs to the same genus as rainbow trout. The cells were cultured in the same growth medium as that used for HEK-293 cells but at 20 °C. Transfection was performed at ~80% confluency using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the protocol established by the manufacturer for primary murine embryonic fibroblast cells. However, we found that using 3 μl instead of 4 μl/assay of the transfection reagent improved the efficacy. Transfected cells were incubated at least for 48 h prior to analysis.

2.7. Confocal microscopy

Cells were transfected with vectors expressing trout ST2-AsRed2 and MYD88-GFP to determine the spatial distribution of the factors. Forty eight hours after transfection, the nuclei of live cells were stained with 1 μg/ml Hoechst 33 342 for 30 min (Sigma-Aldrich) shortly before live imaging.

For immunohistochemistry, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min and permeabilised with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min. An anti–human lamin A/C-specific antibody (1:100; Cell Signalling Technology Inc.) was used to target the nuclear membrane and subsequently stained with an Alexa 488 tagged secondary antibody (1:100; Invitrogen/Thermo Fisher Scientific). Finally, cells were embedded in Fluoroshield (Sigma-Aldrich).

Cells were analysed with the confocal microscope LSM 780 (Carl Zeiss Microscopy), equipped with a 63 × oil-immersion DIC objective and an incubation chamber to maintain temperature (37 °C or 20 °C) and CO_2 (5%) constancy during live imaging. Images were analysed using the software ZEN 2011 (Carl Zeiss Microscopy).

2.8. Nuclear extracts and western blotting

Cytoplasmic and nuclear extracts were prepared from HEK-293 cells. They were collected in ice-cold PBS (1000 × g, 5 min) and the cell pellets were resuspended in ice-cold buffer A (250 μl; 10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA, 1 mM PMSF, 1 mM DTT) containing protease inhibitors (1 mM PMSF; 20 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail, P8340, Sigma-Aldrich). After incubation on ice for 5 min the cell suspension was supplemented with NP-40 (fc 0.5%; Sigma–Aldrich) and gently shaken on ice for 1 min. Nuclei were pelleted (4 °C, 10 000 rpm, 1 min) and boiled for 3 min in 250 μl of Laemmli sample buffer, while the supernatant was mixed with an equal volume of double-concentrated Laemmli buffer and also boiled for 3 min. Aliquots of both extracts were run out on 10% gels and used for Western blotting, essentially as described by Shi et al. (2010).

The GFP-specific primary antibody (A-11122; Life Technologies) at 1 μg/ml targeted GFP-coupled MYD88 from trout and was visualised with a secondary HRP-coupled IgG antibody (Cat. No. 7074; Cell Signalling, Danvers, MA, USA) at a 1:5000 dilution. Blots were developed with an ECL substrate (Bio-Rad, Hercules, CA, USA) and the luminescence was recorded in a ChemiDoc imaging station (Bio-Rad).

2.9. Statistical analyses

Significance of group-specific different mRNA and RLU levels were assessed with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test as provided by GraphPad Prism® v5.01 software package.

3. Results

3.1. ST2L, but no other variant of this factor is encoded and expressed in trout

We retrieved a trout *IL1RL1*-like cDNA (deposited in GenBank as **NM_001281424**), including a 1.8-kb ORF. In the following, we refer to the encoding gene and that factor as ST2, in keeping with the pertinent literature. Our cDNA encodes the full-length factor, also described in literature as *ST2L*. The cDNA sequence suggests that the trout ST2 factor comprises 599 aa residues (GenBank: CCT61382), if the conceptual translation of this sequence commences at that ATG start codon, which had been used for the previously published ST2 protein sequence from salmon (Stansberg et al., 2003). However, we note that the trout and the salmon cDNA sequence feature 21 (trout) and 27 (salmon) nucleotides upstream another ATG codon. Hence, the ST2 factor from trout may in fact be composed of 606 aa residues.

Given the variants of the tetrapod ST2 factor (ST2V, ST2LV, sST2) we scrutinised the detection of *ST2* splice variants in trout. To this end, we distributed 10 different primers across the entire cDNA sequence and used various combinations to amplify fragments of ST2-encoding messages in RT-PCR. Total RNA preparations from a collection of different tissues (liver, spleen, head kidney, gills) were used as templates for cDNA generation. We only found amplicons complying with our original *ST2* variant of this factor (data not

respectively. Identical aa residues between trout and human ST2 are labelled with asterisks; colons and lowercase letters indicate chemically equivalent aa residues. An orange frame indicates experimentally verified interacting proline residue (Brint et al., 2004). Sequence positions are indicated. (**C**) **Phylogenetic relationship of vertebrate TIR domains**. Evolutionary analyses with MEGA5 were based on the aa sequences of TIR domains from seven ST2 (red shaded), eight MYD88 (green shaded) and five TLR factors using the TIR-NBS-LRR class disease resistance protein from *A. thaliana* as an out-group. Sequences from rainbow trout (*O. mykiss)* are underlined. The NCBI accession numbers of the sequences used to generate the neighbour-Joining tree are listed in brackets. Bootstrap confidence values above 50 are indicated representing the percent frequency of appearances of each clade in 1000 replicas. The scale bar represents a genetic distance of 0.2 aa substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

shown).

Sequencing the encoding gene as an alternative approach to detect gene variants gave only evidence of a single gene encoding ST2 in the trout genome (HG325725). The receptor-encoding region is distributed across 11 exons (Fig. 1A); all intron-exon boundaries follow the 5′-GT-3′-AG rule, and the DNA sequence of the conceptually spliced message fully complies with our initial cDNA sequence. The 10 introns are relatively short, varying between 95 and 549 nucleotides in length. In comparison, the *ST2* gene from other vertebrates includes only nine introns (hence 10 exons) within the coding region (*Homo sapiens*, ENSEMBLE transcript ID: **ENST00000233954**; *Mus musculus*, **ENSMUST00000097772**; *Gallus gallus*, **ENSGALT00000030235**), coelacanth *Latimeria chalumnae*, **ENSLACT00000001673**, at least four of which are considerably longer than 1 kb in all those species.

3.2. Low overall conservation of vertebrate ST2 orthologs but co-evolution of the aa residues contributing to the ST2-MYD88 heteromerisation

The aa sequence homology of the trout ST2 receptor is high (94% identity) towards its ortholog from salmon (*Salmo salar)* but low compared with its mammalian counterparts (*H. sapiens*, 28%; *M. musculus*, 26%, *cf.* Table 1). A comparison of the aa sequences of the full length ST2 factors among the vertebrates indicated on one hand considerable differences in their lengths, spanning from 599 aa residues in trout to 533 aa residues in chicken (*G. gallus*). On the other hand, alignments revealed a low aa sequence similarity even among fish species (40%–42% towards *Danio rerio* and *Oreochromis niloticus*; Table 1). Despite this relatively low degree of sequence homology of the whole factor, software programs identified ST2-characteristic functional domains (Fig. 1A). These included a signal peptide (aa positions 1–26), immunoglobulin (Ig) or Ig-like domains (aa positions 68–365; possibly segmented into two distinct subdomains), a transmembrane region (aa positions 368–390) and a C-terminal TIR domain (aa positions 412–589). The latter includes three highly conserved signature motifs (O'Neill and Dinarello, 2000; Suppl. Fig. 1). The protein sequence of the TIR domains reveals the highest degree of sequence conservation from among all the domains of the ST2 receptors indicating significant homology across all the vertebrate sequences (Table 1; Suppl. Fig. 1). Yet, the TIR domains from trout and human ST2 factors share only about 38% aa identity. This unusually low degree of conservation of the ST2-TIR domains is in part due to an insertion of 25 aa residues found in the central part of the salmonid ST2 TIR domain, which is not found in their mammalian orthologs and not entirely conserved among the TIR domains from ST2 factors encoded by fish (Suppl. Fig S 1).

The entire TIR domain of the trout ST2 factor is encoded by exons 10 and 11 (Fig. 1A). Segmentation of the TIR domain of ST2 factors on two exons is similarly found in all known ST2-encoding genes, as revealed by analysing the exon segmentation of the ST2 TIR domains from human, mouse, chicken (data not shown).

It is known from mammals that ST2 and MYD88 may heteromerise through direct physical contact of their TIR domains. Projecting the documented contact sites for the heteromerisation of human ST2L and MYD88 factors (Basith et al., 2011) onto the respective trout sequences reveals that a string of approximately 30 aa residues features almost precise factor-specific spatial conservation of those aa residues known to shape the contacting interfaces (Fig. 1B; Suppl. Fig. S1). The conserved residues G458 of trout ST2 and R183 of trout MYD88 may form a hydrogen bond, while the charged residues of ST2 (D424, E447, R459, D460, D461) and MYD88 (H163, E170, D182, R183, D184) may allow for electrostatic interactions. Conserved within this area is also a proline residue (P462 of the trout sequence; Fig. 1B), which was shown by point mutation of the murine factor to be absolutely required for ST2-MYD88 heteromerisation (Brint et al., 2004).

3.3. Factor-specific conservation of the TIR domains from ST2 and MYD88

Based on its cytoplasmic TIR domain, the ST2 factor from salmon was previously sorted in proximity to the ST2 factors from other vertebrates and separated with some probability (<70%) from other factors of the IL1R/TLR superfamily (Stansberg et al., 2003). This previous phylogenetic analysis included only the salmon ST2 sequence as a representative for the teleostean orthologs, because no other ST2 from fish was known at that time. We now repeated this analysis, included the ST2-TIR sequences of five fish species and recalculated a phylogenetic tree of the TIR domain–containing factors ST2, MYD88 and TLRs (Fig. 1C). In vertebrates, the superfamily of these factors is generally subdivided into (i) TLRs, characterised by extracellular leucine-rich repeats; (ii) interleukin receptors, characterised by extracellular Ig domains; and (iii) adaptor proteins of the TLR and IL1R signalling pathways. Our phylogenetic tree reflects this subdivision. MYD88 and ST2 clusters are clearly separated into subclades comprising sequences from tetrapods and teleosts, respectively, with a bootstrap confidence level of 100%. The rooting of the species within the clusters of ST2 and MYD88 factors is all in agreement with the respective expectations: the salmonid factors are sorted together, and the fish factors are distinct from higher vertebrates.

3.4. Multiple alignments and gene segmentation also reveal the common ancestry of the N-terminal part of ST2

The low degree of conservation of the N-terminal part of ST2 sequences raised doubts about its decent from a common primordial gene. We therefore aligned the respective segments of the ST2 sequences from trout and human towards that of chicken. The latter was included as an evolutionary intermediate since the aa sequence of the salmonids is so divergent from that of mammals that a convincing homology is not immediately apparent. The alignment reveals many short amino acid sequence motifs almost perfectly conserved between all three or either of the species (Fig. 2). Perfectly conserved have been 54 aa residues (from among 372), and additional 114 residues have been preserved as chemically equivalent aa. An even stronger support for the common ancestry of this part of the ST2-encoding genes comes from projecting the segmentation of the trout and human genes onto the ST2 factor and its domains (Fig. 2). The exon boundaries downstream from exon 2 of the trout gene (exon 3 of the human gene) are all almost coinciding. The transmembrane domain is similarly encoded by an own exon (exon 9, trout), as are the TIR domains (exons 10, 11; Suppl. Fig. 1). Moreover, this alignment reveals that the trout factor (similar as that from salmon) features an insertion of 30 contiguous aa residues which are all encoded on the 5' terminus of exon 3. This insertion is not found in chicken and human. The data altogether validate the common ancestry of the ST2-encoding genes from Teleosteii to mammals.

3.5. Expression of ST2 and MYD88 genes is constitutive in embryonic, larval and adult rainbow trout and not regulated during infection

We profiled the modulation of the mRNA abundances of *ST2* and *MYD88* from early developmental stages until shortly after hatching and compared these data with the levels of factor expression found in several tissues of approximately 1-year-old rainbow trout.

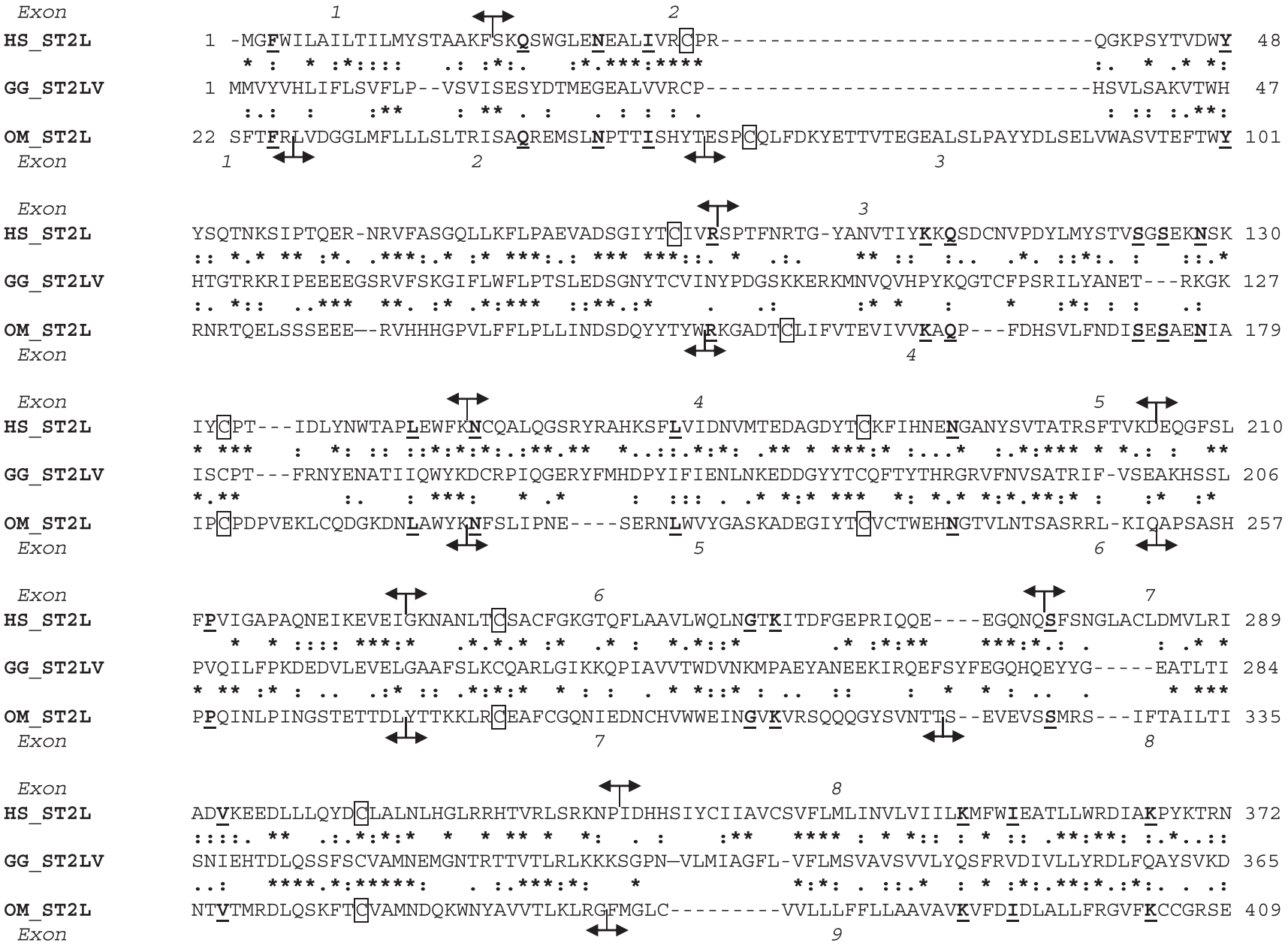

Fig. 2. Common ancestry of the vertebrate ST2-encoding gene. The aa sequences of the N-terminal part of human, chicken and trout ST2 factors have been aligned, and the exon segmentation of the human and trout ST2-encoding genes was projected onto the sequences. Exon numbers are given above the sequences. The aa positions of the factors are indicated. Asterisks denote identical aa residues, while colons indicate chemically equivalent residues. Bold and underline indicate residues being identical between trout and human but divergent from chicken. Arrows indicate exon boundaries. Boxed are cysteine residues known to contribute to folding the Ig/Ig-like domains. Dashes indicate gaps introduced to optimise the alignments.

Moreover, we were interested to learn whether the expression of these factors would be modulated after infecting adult fish with the pathogen *Aeromonas salmonicida*.

Considering the embryonic and larval stages, we found that *ST2* and *MYD88* mRNA abundances were clearly highest at the earliest embryonic stage (day 21 postfertilisation) and declined significantly during ongoing development. The mRNA abundances of both factors were of similar levels at the individual time points during embryonic and larval development (Fig. 3A). In adults, however, the mRNA concentrations of both factors were significantly disproportioned in several tissues. The abundance of the *ST2* mRNA was found to be quite similar in spleen, gills, trunk kidney and head kidney and slightly lower in brain and muscle (Fig. 3B). Yet the concentrations of the *MYD88* mRNA were much and significantly higher in spleen, gills and head kidney than those in other tissues, clearly exceeding by severalfold the concentrations found in liver and trunk kidney. Hence, the concentrations of the *MYD88* mRNA exceeded those of the ST2-encoding factor by severalfold in the immune-relevant tissues spleen, gills and head kidney while they were at similar or even lower levels in the other tissues.

Infection may modulate the tissue-specific copy number particularly of immune genes because of altered transcription and/or cell migration and/or proliferation. We therefore quantified ST2-encoding transcripts in spleen, gills, and head kidney of trout at 6 h, 12 h and 72 h after infecting healthy fish with 1×10^7 viable *A. salmonicida* bacteria. However, the copy number of ST2-encoding mRNAs showed no significant infection-related alterations (Fig. 3C).

3.6. ST2 quenches pathogen-driven TLR signalling in HEK-293 cells

We examined the role of ST2 from trout in TLR-mediated pathogen signalling. TLR signalling ultimately activates the NF-κB-transcription factor complex (Kawai and Akira, 2007), and hence, stimulus-dependent NF-κB activation reflects TLR signalling. Therefore, human HEK-293 cells were cotransfected with a vector expressing the bovine *TLR2* together with a reporter gene under the control of a modified NF-κB-responsive ELAM promoter and added increasing amounts of our trout *ST2* expression vector into the transfection cocktail. Half of the cultures were kept as unstimulated controls, while TLR2 signalling was stimulated in the other dishes by the addition of 30 μg/ml of heat-inactivated *E. coli*$_{1303}$ bacteria. Transfecting increasing amounts of the trout ST2 expression construct did not modulate the basal NF-κB activity compared with the untransfected controls (Fig. 4A, grey columns). However, increasing amounts of the trout ST2 expression construct resulted in dose-dependent lowering of the TLR2-mediated NF-κB activation in pathogen-challenged cultures, from 7.1 ± 0.2–fold in stimulated cells without overexpressed trout ST2 down to 2.0 ± 0.4–fold in cells, having received 4 μg of the trout ST2 expression vector (Fig. 4A, red symbols and line). In a complementary experiment, we used an NF-κB-activated trout SAA promoter construct (Rebl et al., 2011b) as an alternative to monitoring TLR2 signalling and observed again that ST2 quenched the promoter activation under the same experimental conditions (Fig. 4B). The TLR2-mediated 2.4 ± 0.2–fold stimulation of the SAA-promoter activity was entirely blocked by transfecting as little as 0.5 μg of the trout ST2 expression vector. This ST2 effect can solely be attributed to the transfected ST2 expression vector since HEK-293 cells do not express endogenous ST2 (Shaw et al., 2002).

3.7. Localisation of overexpressed ST2 and MYD88 in human HEK-293 cells

ST2 is thought to exert its dampening role in TLR signalling by prohibiting MYD88 recruitment to the cytoplasmic TIR domain of transmembrane TLR2 or TLR4 receptors (Brint et al., 2004; Guven-Maiorov et al., 2015a). Hence, we analysed the subcellular distribution of overexpressed trout ST2 factors and wondered whether the presence of this factor would alter the distribution of MYD88 factors. We tagged the trout ST2 factor with the red fluorescent protein AsRed2 and MYD88 with the green fluorescent protein GFP and expressed them in human HEK-293 cells. Confocal laser scanning

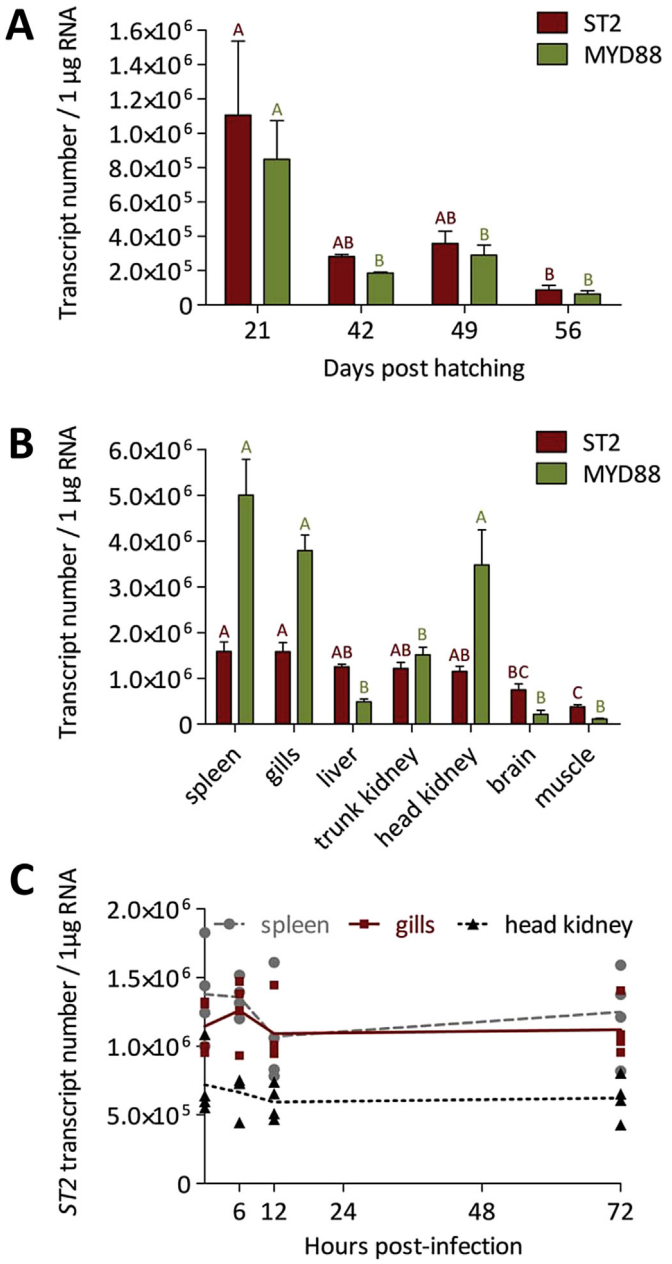

Fig. 3. ST2 and MYD88 expression levels during development, in different tissues and during infection. Quantitative RT-PCR analysis of *ST2* (red columns) and *MYD88* (green columns) transcript levels in **(A)** trout embryos or larvae ($n = 4$) and **(B)** selected tissues of adult trout ($n = 8$). Columns represent the mean relative copy numbers of the target gene (error bars, SEM), calculated per 1 μg of total RNA used as template for cDNA generation. Different letters above the bars indicate significant differences ($p < 0.05$). **(C)** ST2 mRNA concentration in different tissues from trout after infection with *A. salmonicida*. Concentration of ST2 transcripts in spleen (grey dashed line, circular symbols), gills (red full line, squared symbols) and head kidney (black dotted line, triangles) at various time points after infection (abscissa, $n = 4$ for any time point). Lines were fitted to the mean values (ordinate, transcript numbers per 1 μg total RNA) at any one time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

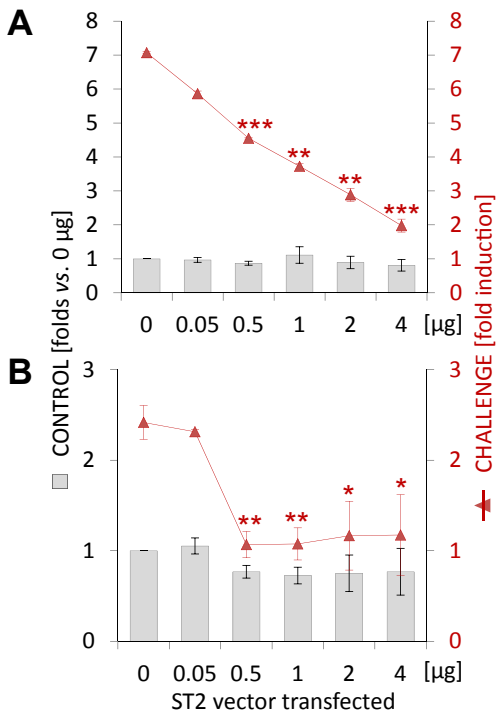

Fig. 4. ST2 quenches pathogen-stimulated TLR2 signalling. HEK-293 cells were cotransfected with constructs expressing bovine *TLR2* and (**A**) the mammalian *ELAM*-driven luciferase expressing NF-κB reporter or (**B**) a luciferase expressing reporter plasmid containing the trout SAA promoter fragment together with increasing amounts of the *ST2* expression vector, as indicated (abscissa). Columns indicate fold changes of the luciferase activity relative to the control, having received no ST2 expression vector and no stimulation (control, left ordinate). Line graphs (red) show the fold change of luciferase activity after stimulating the cells for 24 h with 30 µg/ml inactivated *E. coli* 1303 particles, relative to the unstimulated control. Statistical significance compared with the control group was assessed using one-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microscopy revealed that the hosting vectors for both fluorescent proteins were expressed in the HEK-293 cells and resulted in a uniform cellular distribution of either of the fluorescent proteins with no signs of any spatially restricted localisation (Fig. 5A1, B1).

ST2 distribution: The ST2-AsRed2 tagged protein always very prominently localised at the nuclear membrane and in a sacculus of the endoplasmic reticulum extending from the nuclear membrane into the cytoplasm (Fig. 5A2). The latter structure always covered less than 20% of the cytoplasm as judged from more than 50 images derived from independent transfection assays with different plasmid preparations. A single thick heavily fluorescent inclusion body of the overexpressed protein was also found in almost any of those cells (Fig. 5A2). The high concentration of the ST2-AsRed2 protein at the nuclear membrane is quantitatively reflected by the distribution of the fluorescence intensities as measured across the cells (Fig. 5A3). The highest intensities of the red fluorescence always flanked the boundary of the blue fluorescence stemming from DNA counterstaining with the Hoechst 33 342 dye. In order to exclude that the usage of the particular AsRed2-coupled vector directed ST2 into the nuclear membrane, we inserted ST2 together with AsRed2 into the pAM505 plasmid backbone (the same vector used for trout MYD88 expression) and found the same localisation of ST2 as seen before (Suppl. Fig. S2A).

We never observed an association of ST2-AsRed2 itself with the cell membrane. To validate this surprising result, we co-expressed this factor with GFP-tagged SLC6A8 (solute carrier family 6 member 8) from trout. This factor is known to integrate into the cell membrane (Borchel et al., 2014; Zorzano et al., 2000). The images unambiguously show no co-localisation of both proteins (Fig. 5A4). In addition, we demonstrated by co-staining with *anti*-lamin antibodies, a broadly used marker for nuclear membranes (Jost and Johnson, 1981), that the localisation of ST2 and lamin is congruent (Fig. 5A5).

MYD88 distribution: GFP-tagged MYD88 was always distributed throughout the cytoplasm, frequently accumulating in cytoplasmic aggregates (Fig. 5B2). Significantly, MYD88-GFP was excluded from the nucleus, unlike the GFP protein alone. This impression gathered from the microscopic images of those cells is very clearly validated by profiling the fluorescence intensities across the cells (Fig. 5B3).

MYD88 distribution in the presence of overexpressed ST2: Expressing both fluorescent-tagged proteins unexpectedly allowed MYD88-GFP to enter into the nucleus (Fig. 6A1). This was invariantly seen in all the cells expressing both proteins (>50 images) and clearly revealed by the intensity profiles recorded across the cells (Fig. 6A2). Co-expression of both factors resulted in an approximately uniform cytoplasmic and nuclear distribution of MYD88. However, one week after transfection MYD88 was no longer diffusedly distributed across the nuclei but rather concentrated in few aggresome-like structures within them (Suppl. Fig. S2B).

We validated in Western blots the nuclear localisation of MYD88 in those cells expressing ST2 together with MYD88 (Fig. 6A3). To this end, HEK-293 cells were stably transfected with expression plasmids for either trout MYD88-GFP or ST2-AsRed2. Some of those cells stably expressing ST2 were in addition transiently transfected with the MYD88-encoding plasmids. Nuclear and cytosolic extracts were prepared from both, MYD88-expressing control cultures and those co-expressing ST2 and MYD88. Following Western blotting, we detected MYD88-GFP with a GFP-specific antibody as a clear, single band with a molecular weight of approximately 60 kDa in agreement with the calculated molecular weight of GFP-tagged trout MYD88 (Fig. 6A3, right-hand panel). MYD88-GFP was found in the cytosolic extracts of all cells. However, it was only found in nuclear extracts of those cells co-expressing ST2 and MYD88. This result validated the laser-scanning-based microscopic observations.

3.8. Validation of the spatial distribution of ST2 and MYD88 in trout CHSE-214 cells

We repeated the coexpression experiments using the salmonid derived CHSE-214 cell line in order to evaluate whether interspecies incompatibilities between the mammalian host cell and the fish factors may have caused the unexpected ST2 localisation as well as its effect on the spatial distribution of the MYD88 factor. We found the same spatial distributions of both factors as seen in the HEK-293 cells, including (i) a prominent restriction of ST2 to the nuclear membrane (Fig. 6B1), (ii) the exclusive cytoplasmic distribution of the trout MYD88-GFP factor (Fig. 6B2) and (iii) an approximately uniform cytoplasmic and nuclear distribution of MYD88-GFP in those cells coexpressing ST2 and MYD88 together (Fig. 6B3, 6B4). The ST2 effect can solely be attributed to the transfected ST2-AsRed2 expression vector since these cells do not endogenously express ST2 (Suppl. Fig. S3).

4. Discussion

The mammalian ST2 factor (alias IL1RL1) is known to have three seemingly unrelated different functions. It (i) promotes cell growth and multiplication (Tominaga et al., 1989; Tominaga et al., 2015), (ii) serves as the receptor for IL33 (Haraldsen et al., 2009; Schmitz et al., 2005) and (iii) quenches TLR-mediated signalling by sequestering the TLR-adapter MYD88 (Brint et al., 2004). Already the first superficial comparative DNA sequence analysis of our cDNA sequence retrieved from trout revealed a high degree of sequence homology

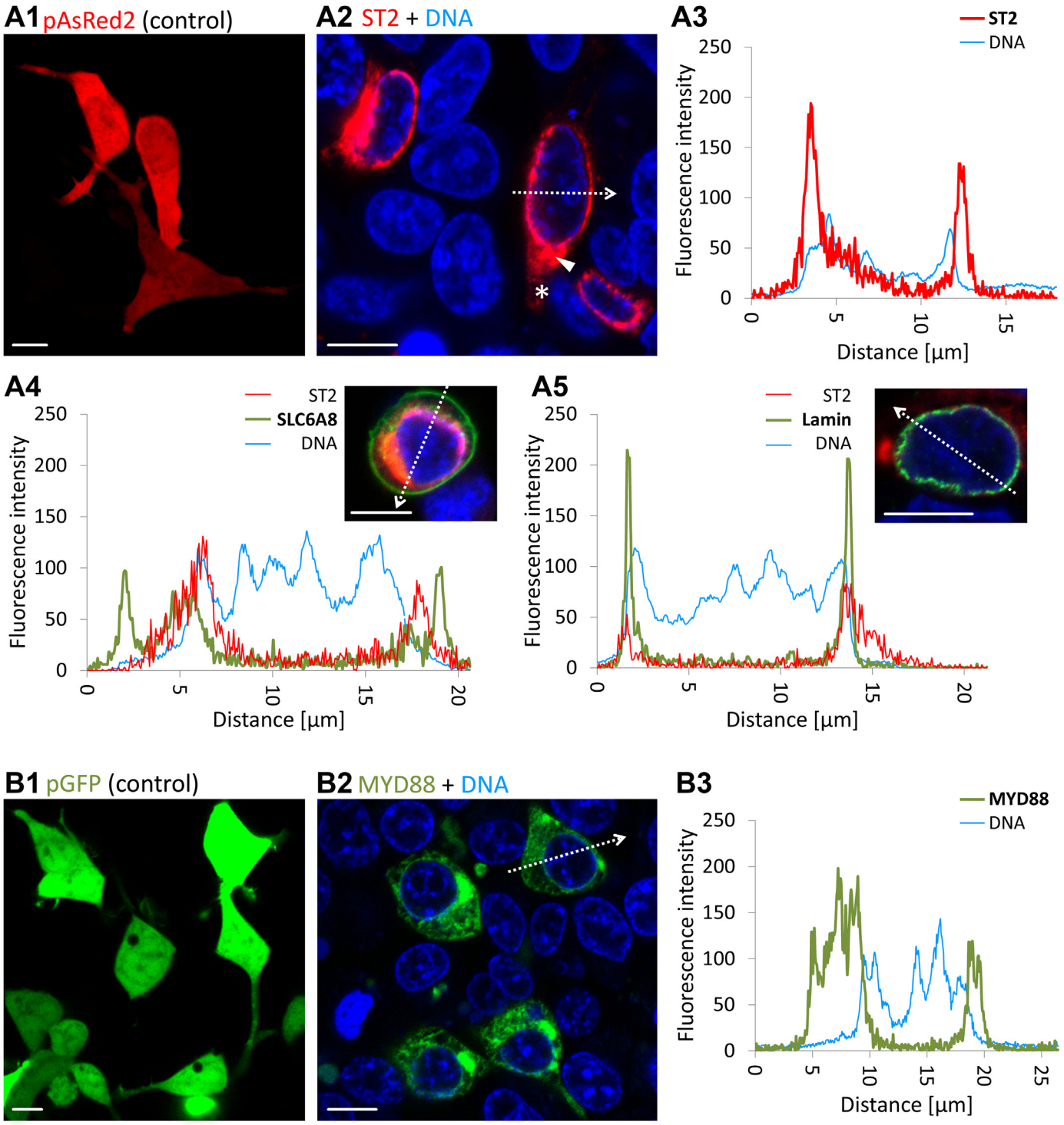

Fig. 5. Subcellular localisation of trout ST2 and MYD88 factors. Human HEK-293 cells were transfected with plasmids expressing GFP or AsRed2 alone (**A1**, **B1**; control) or the tagged proteins ST2-AsRed2 (**A2**; ST2) or MYD88-GFP (**B2**; MYD88). Cells were visualised 24 h after transfection with a confocal laser scanning microscope. Nuclei were stained with Hoechst 33342 (DNA). Scale bars represent 10 µm in all scanning images. (**A2**) ST2 was localised (ST2 + DNA) in or at the nuclear membrane and in a netlike arrangement around a sacculus emanating from the nuclear membrane (asterisk) and in mostly a single heavily staining inclusion body (arrow). (**A3**) Profile of fluorescence intensities (ordinate) recorded at specific locations (abscissa) across the cell following that path as indicated by the dotted arrow in panel A2. (**A4**) HEK-293 cells cotransfected with constructs expressing ST2-AsRed2 and a GFP-tagged SLC6A8 (a marker for the cell membrane) from trout. (**A5**) HEK-293 cells were transfected with the trout ST2-AsRed2 expression vector, and lamin (a marker for the nuclear membrane) was detected with a specific antibody (green fluorescence). Note the virtual absence of any red fluorescence at the cell membrane in panel A4, but coincidence of red and green fluorescence (nuclear membrane) in panel A5. (**B2, B3**) Cytoplasmic but not nuclear distribution of GFP-tagged trout MYD88; same setting as shown in A. The selected images are representative for >50 images taken in at least three independent experiments using different plasmid preparations of each of the different vectors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

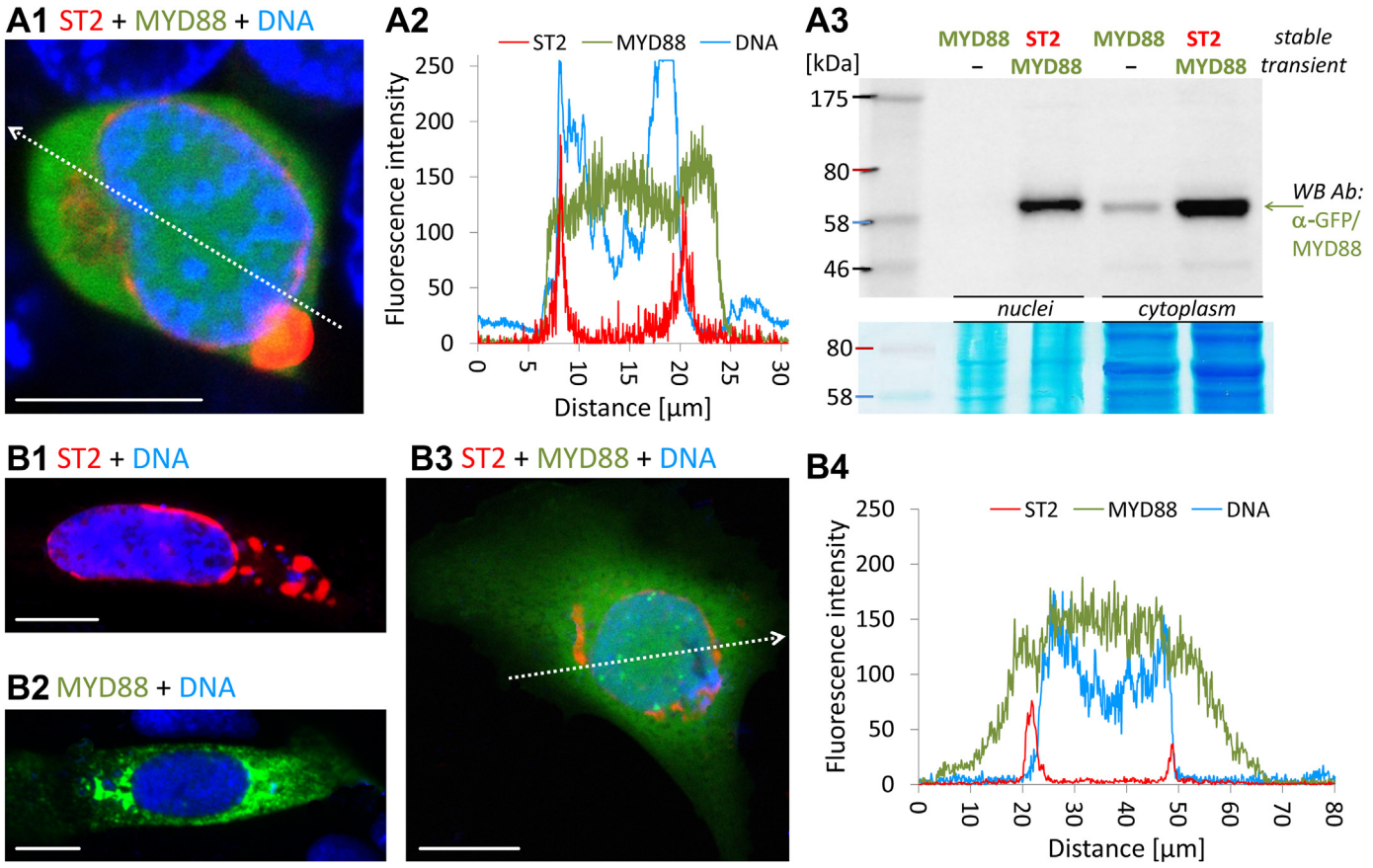

Fig. 6. Coexpression of trout ST2 and MYD88 factors allows the nuclear translocation of MYD88 in HEK-293 and trout CHSE-214 cells. (**A1**) Coexpression of ST2 and MYD88 results in uniform cytoplasmic as well as nuclear MYD88 distribution. **(A2)** The intensity profile reveals approximately the same intensity of the green fluorescence in the nuclear area (distance 9–21 µm) as in the cytoplasm (distance 21–26 µm). (**A3**) Western blot resolving GFP-tagged MYD88 of nuclear or cytoplasmic fractions of HEK-293 cells having stably been transfected with the trout MYD88-GFP or ST2-As2Red expression vector (upper line, stable) and which had in addition transiently been transfected with the MYD88 expression vector (lower line, transient). The Coomassie-stained protein gel shown below served as a loading control. No nuclear MYD88-GFP was detected in the Western blots of the solely MYD88 transfected controls, but it was very clearly detected in the ST2-MYD88 doubly transfected cultures. This experiment is representative of three. (**B1**) ST2-or (**B2**) MYD88-transfected CHSE-214 cells reveal the same spatial distribution of the respective factors as seen in the HEK-293 cells (cf. Fig. 4A2, B2). (**B3, B4**) Cotransfection of both expression vectors into CHSE cells also resulted in the nuclear translocation of MYD88. Scale bars represent 10 µm in all images. All images are representative of at least 50 transfected cells per condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

towards the respective factor from salmon but low similarity to ST2-encoding sequences from higher vertebrates. Based on the cDNA sequence of the salmon factor (Stansberg et al., 2003), it was suggested that it would feature three Ig-like domains and a TIR domain, as is common in the other ST2 factors. Identification of the Ig-like domains was solely anchored at the conserved positioning of four from among six cysteine residues known to be crucial for shaping Ig-like domains 2 and 3 of the mammalian ST2. Moreover, the phylogenetic analysis grouping this factor into the vicinity of ST2 factors from higher vertebrates was only based on the better conserved cytoplasmic TIR domain of the factor.

IL33 is not known to be expressed in bony fish (Ogryzko et al., 2014), hence making it unlikely that Teleosteii encode—let alone express—a receptor for IL33. Low sequence similarity of the N-terminal (putative ligand binding) part of the factor together with questionable common functions made us doubt whether this factor from fish does really represent an ortholog of the mammalian factor.

4.1. Ancient origin of the ST2-encoding gene

We revealed significant homology of the TIR domain sequence of the trout ST2 receptor towards its counterparts from higher vertebrates, and they all are encoded on two exons. This undoubtedly demonstrates their common origin. However, this does not necessarily imply that the other parts of the ST2 factor stem from a common ancestor. Rather, TIR domains may have been swapped into different genes. The TIR domain is of bacterial origin and was spread into some genes of the Plantae and Animalia kingdoms of the eukaryotes (Beutler and Rehli, 2002). Examples indicating the swapping of the TIR domain into different gene families are the TLR gene family or the different members of the IL1R subfamily. Both gene families contain TIR domains, but the ligand-binding N-terminal parts of these genes are entirely unrelated (Boraschi and Tagliabue, 2013; O'Neill, 2008).

For tracing back the origin of the N-terminal 2/3rd of the ST2 factor—known in mammals to bind IL33—we had to include the sequence from chicken into the multiple alignments to recognise some short but obviously conserved motifs of the protein sequences. Aves represent an evolutionary intermediate. Bony fish species diverged more than 400 million years ago from the lineage leading to mammals, while birds branched off approximately 290 million years ago (Hedges et al., 2015). Overall, we observed a gradual divergence of the N-terminal part of the ST2 factor over that extended evolutionary timescale. However, the most compelling evidence for the common ancestry of the ST2-encoding genes was revealed by projecting the exon segmentation of the trout and human ST2-encoding genes onto the protein sequences. The gene structure of those areas encoding Ig-like domains 2 and 3

of the ST2 receptor is well conserved between human and trout. Hence, ST2-encoding genes from teleost fishes and higher vertebrates are structurally true orthologs.

4.2. ST2 from trout is a negative regulator of TLR signalling

The constitutive expression of *ST2* in embryonic, larval and adult trout indicates a vital function of this factor throughout the life cycle. Its expression was not modulated in a variety of organs during infection of fish with the Gram-negative *A. salmonicida* pathogen, complying with similar observations made after challenging salmon with LPS (Stansberg et al., 2003).

We probed the ST2-mediated dampening of TLR signalling as the only one from among the three different functions attributed to the mammalian ST2 factor. We show in the HEK-293 reconstitution system of TLR signalling that ST2 from trout reduces in a dose-dependent fashion the TLR2-mediated activation of two unrelated promoters (SAA from trout and ELAM from human). Activation of both promoters requires increased levels of NF-κB factors (Rebl et al., 2011b; Yang et al., 2006). The pathogen-induced activation of the SAA promoter was more sensitive to the quenching effect of ST2 than that of the ELAM promoter. This likely reflects that the SAA promoter of the reporter construct features only a single NF-κB attachment site (Rebl et al., 2011b), while the genetically engineered ELAM promoter harbours five of these sites (Schindler and Baichwal, 1994). Our data conclusively validate that trout ST2 is a negative regulator of TLR2 signalling in HEK-293 cells by dampening NF-κB activation, similar as observed to using overexpressed ST2L from mouse (Liu et al., 2010).

Interestingly, *ST2* overexpression did not modulate the basal level of NF-κB factors in the HEK-293 cells. Using identical experimental settings, we have previously reported that transfecting increasing amounts of constructs expressing trout *IRAK4* (Brietzke et al., 2014) or trout *TLR2* (Brietzke et al., 2016) significantly and strongly reduced the threshold level of active NF-κB factors in these cells, suggesting the interference of these factors with the turnover of active NF-κB factors in this mammalian cell. Moreover, transfecting a vector expressing the trout *MYD88* will increase the level of active NF-κB factors in these cells (Brietzke et al., 2016). These observations altogether show that transfecting such fish factors known to physically engage into the signalling platform around the TIR domain signalosome (Guven-Maiorov et al., 2015b) may directly influence the steady-state levels of NF-κB factors. This is conceivably mediated through their TIR domains. While the role of the TLR signalosome in maintaining the homeostasis of active NF-κB factors in HEK-293 cells is unknown, clearly the ST2 factor from trout does not contribute to this process. Conceivably, its TIR domain executes only a subset of the various TIR functions which may be exerted by TIR domains of other factors. Certainly, preserved in the trout ST2-TIR is its interaction with MYD88 since the identity and arrangement of those amino acids known to physically interact with this factor have clearly been conserved, from fish to mammals. Other TIR-related functions may not have been preserved as indicated by the comparably low degree of overall conservation of the aa sequence of the ST2-TIR domain. In contrast, the very high degree of conservation of the TIR domain from MYD88 factors both within a species (Fornarino et al., 2011) and during evolution (Singh et al., 2014) conceivably reflects the multiple functions of this factor. It interacts at least with the TIR domain of receptors (TLRs, IL1Rs), kinases (IRAKs), adapters (MYD88, TRIF; Brikos and O'Neill, 2008; Kawai and Akira, 2007) and transcription factors (IRFs; Kawai et al., 2004).

4.3. Trout ST2 exerts its function not at the cell membrane

Mammalian ST2 quenches TLR signalling by interacting with MYD88 *via* their TIR domains (Brint et al., 2004). Activated TLRs recruit MYD88 into the Myddosome (De Nardo, 2015; Lin et al., 2010) as clearly documented in confocal microscopy (Kagan and Medzhitov, 2006). Hence, we wanted to use this technique to visualise that ST2 localises at the cell membrane and assumed that it may concentrate MYD88 at that location in a function-dependent fashion. We have previously validated that the bovine TLR2 as expressed from that vector used in the current study indeed localises at the membrane of HEK-293 cells (Yang et al., 2008). Surprisingly, we found that our tagged trout ST2 localised at the nuclear membrane and apparently in some saccule-like protrusion of the endoplasmic reticulum. This was similarly observed in human HEK-293 and salmonid CHSE-214 cells. We did not find a single cell revealing ST2 association with the cell membrane. Hence, our ST2 factor could not have exerted its function to dampening TLR2 signalling at or around the cell membrane.

Nuclear-rather than cell-membrane-associated localisation of ST2 is at variance with current assumptions regarding the ST2 localisation in mammalian cells. Recent reviews all discuss that ST2 represents a transmembrane protein being recruited to cell membranes and reaching into the intracellular space to receive external signals (Basith et al., 2011; Guven-Maiorov et al., 2015a; Kakkar and Lee, 2008; Liew et al., 2005). However, previous studies show that only very few cell types express ST2 on the cell surface. For example, $CD4^{+}NK1.1^{-}$ but neither $CD4^{+}NK1.1^{+}$ nor $CD4^{-}NK1.1^{+}$ nor dendritic cells from spleen express ST2 on their surface (Löhning et al., 1998). Abundant expression of ST2 was observed in dendritic cells but not as a cell-membrane-associated factor (Rank et al., 2009). We only found a single report on the localisation of recombinantly expressed ST2. In that study, the human ST2V variant was detected on the plasma membrane of COS7 cells (Tago et al., 2001). Whether overexpressed mammalian ST2L will also localise at the nuclear membrane in our model cells remains to be seen in future experiments.

We recognise that, in mammals the ST2 ligand IL33 is known as a chromatin-associated factor (Carriere et al., 2007; see Oboki et al., 2010; for a review). Hence, it may be envisaged for mammalian species that ST2 residing in the nuclear membrane may somehow help IL33 traverse the nuclear membrane.

4.4. Interaction of the overexpressed factors ST2 and MYD88 likely occurs, but its role during dampening TLR2 signalling is unclear

Our most astonishing observation was that the coexpression of the trout factors ST2 and MYD88 permitted the MYD88 factor to enter the nucleus. Overexpressed MYD88 alone was only found in the cytoplasm and not in the nucleus, as expected from its mammalian (Kissner et al., 2011; Nishiya et al., 2007) and teleost orthologs (Iliev et al., 2011). However, whenever we cotransfected vectors expressing both factors, we found approximately similar concentrations of MYD88 in the cytoplasm and the nucleus. This peculiar ST2-dependent MYD88 distribution was observed in human HEK-293 cells as well as in salmonid CHSE-214 cells. Hence, interspecies incompatibilities cannot account for this unexpected cellular distribution. Our data collectively show that both factors interact since ST2 modulates the localisation of MYD88. Factor interaction conceivably is mediated through their TIR domains since their contacting interfaces have structurally been preserved over long evolutionary times.

The fairly even MYD88 concentration in cytoplasm and nucleus intuitively suggests passive diffusion of the overexpressed MYD88 factor through both compartments. However, it is extremely unlikely that such a big molecule like the GFP-tagged MYD88 factor (MW approximately 60 kDa) could simply diffuse across the nuclear membrane. This would only be possible if the trout ST2 factor

punctured the nuclear membrane. This in turn could lead to cell death. Yet our stably ST2-transfected cells survived for more than 4 weeks. Moreover, these cells neatly concentrated the nuclear MYD88 after several days into aggresomes. Their formation is an active process to inactivate excessive protein loads stemming from overexpression (Iliev et al., 2011).

Nuclear localisation of MYD88 has sometimes been observed. Ultrastructural immunohistochemistry identified a weak abundance of overexpressed MYD88 in the nuclei of many HeLa cells, but only of those cells not displaying signs of apoptosis (Jaunin et al., 1998). Arancibia et al. (2007) found a nuclear localisation and export motif in mammalian MYD88 and concluded that these were crucial for nuclear-cytoplasmic transit when TLR2 is activated. We identified a bipartite nuclear localisation signal sequence within the trout MYD88 protein (CDG03206) at positions 200 to 228, suggesting that trout MYD88 has indeed the structural precondition to be imported into the cell nucleus.

The intracellular distribution of MYD88 depends on the proper structure of the extracellular domain (but not the TIR domain), and displaced MYD88 factors may block TLR signalling (Nishiya et al., 2007). Only N-terminally crippled MYD88 factors enter the nucleus. Hence, one could formally argue that the ST2-mediated quenching of TLR signalling may be brought about by a structural deformation of MYD88 through its interaction with ST2. The structural rearrangement of the MYD88 factor may expose the otherwise hidden nuclear localisation signal. This interaction would need to be short-lived since our images do not show any coaggregation of MYD88 with ST2. Thus, ST2 is unlikely to simply sequester MYD88 at the nuclear membrane to remove it from the active signalling cascade. However, relevance and capacity of such speculations can only be evaluated in future experiments.

5. Conclusions

1. Our evolutionary comparison of the ST2 factor from trout and the architecture of its encoding gene validates the common ancestry of the teleost and tetrapod variants of this gene. The N-terminus of the trout factor is elongated compared with ST2L from tetrapods because of an insertion into exon 3. Exon sizes from exon 4 to 11 have been better preserved during evolution. Salmonid ST2-encoding genes also encountered an insertion into the C-terminal exon encoding the TIR domain. These insertions altogether cause a considerable variation in the factor length.
2. Trout ST2 dampens TLR2 signalling and hence is, by this criterion, a functional ortholog of the mammalian factor.
3. Overexpressed trout ST2 localises at and around the nuclear membrane and causes MYD88 to enter into the nucleus. ST2-dependent alteration of the spatial intracellular distribution of MYD88 indicates some interaction of both factors, conceivably mediated through evolutionary preserved interaction sites of their TIR domains. However, whether and how the peculiar arrangement of ST2 at the nuclear membrane possibly contributes to the ST2-mediated dampening of TLR2 signalling is unclear.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.10.009.

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